



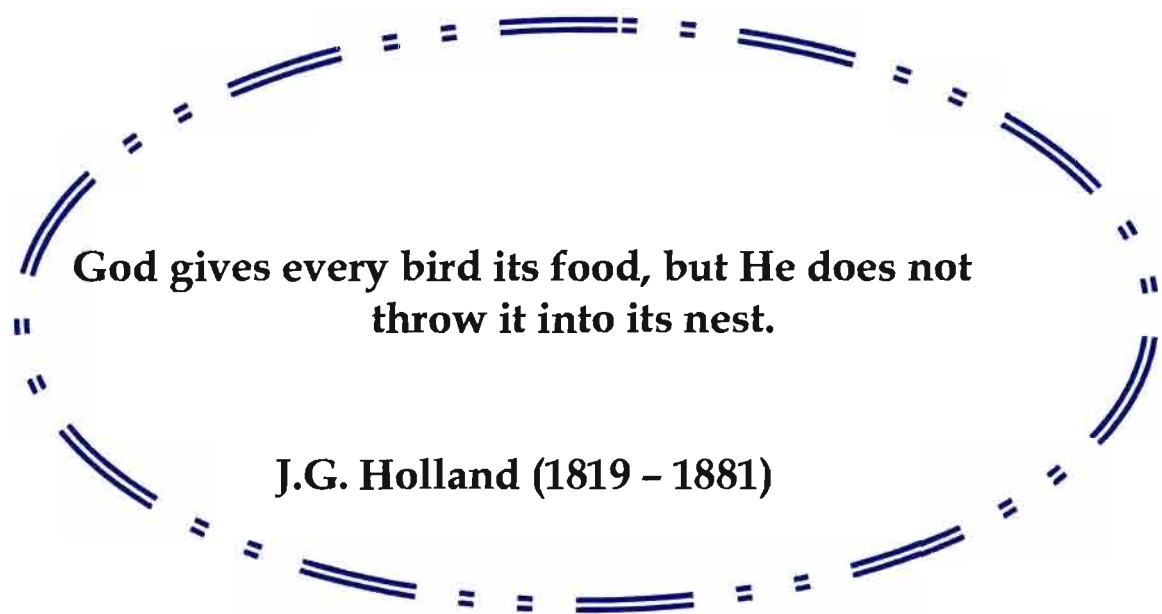
Thesis Presented for the Degree of DOCTOR OF PHILOSOPHY in the Faculty  
of Health Sciences, Department of Medicine, UNIVERSITY OF CAPE TOWN

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**God gives every bird its food, but He does not  
throw it into its nest.**

**J.G. Holland (1819 - 1881)**

*I thank the Almighty GOD for the strength and provision*

*To*

*Rataba, Sefora and Refilwe Tselanyane*

*In Memory of my Papa (Rataba Josiah Tselanyane),  
who passed away three days after this thesis was  
submitted. I thank God for you. Thank you for being a  
loving, caring & supporting father.*

*Ke tla go gopola ka dinako tsotlhe Monareng.*

## Declaration

I, MALEFA LYDIA TSELANYANE hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the substance nor any part of this work has been, is being, or is to be submitted for another degree at this University or any other University.

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This thesis is presented for examination for the degree of Doctor of Philosophy.

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## LIST OF ABBREVIATIONS

APAD	3-acetylpyridine adenine dinucleotide
APADH	Reduced 3-acetylpyridine adenine dinucleotide
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
AR	Analytical reagent(s)
ATP	Adenosine triphosphate
CDCl <sub>3</sub>	Deuterated chloroform
CHO	Chinese hamster ovarian
CHCl <sub>3</sub>	Chloroform
cm	Centimetre(s)
CO <sub>2</sub>	Carbon dioxide
COSY	Correlation Spectroscopy
CQ	Chloroquine
CQR	Chloroquine-resistant
CQS	Chloroquine-sensitive
DCM	Dichloromethane
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DMEM	Dulbeccos eagles modified medium
DMSO	Dimethyl sulfoxide
EtAC	Ethylacetate
FCS	Foetal calf serum
g	Gram (s)
H <sub>2</sub> O	Water
HAMS-F12	Ham's Nutrient Mixture F12
HCl	Hydrochloric acid
HMQC	Heteronuclear multiple bond quantum correlation
HRMS	High resolution mass spectrometer
hrs	Hours



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HSQC	Heteronuclear single bond quantum correlation
HPLC	High Performance Liquid Chromatograph
Hz	Hertz
IC <sub>50</sub>	Inhibitory concentration at 50%
LC/MS/MS	HPLC linked to Mass spectrometer
MeCN	Acetonitrile
MeOH	Methanol
mbar	millibar
mg	Milligram
mg/kg	Milligram/kilogram
mg/ml	Milligram per milliliter
ml	Millilitre
ml/min	Milliliter per minute
mm	Millimeter
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide hydrogen
NMR	Nuclear magnetic resonance
<sup>1</sup> H-NMR	Proton NMR
<sup>13</sup> C-NMR	<sup>13</sup> Carbon NMR
NBT	Nitro blue tetrazolium
nm	Nanometer
NOESY	Nuclear Overhauser Enhancement Spectroscopy
PBS	Phosphate buffered saline
PE	Petroleum ether
PES	Phenazine ethosulfate
pH	- logarithm [H <sup>+</sup> ]
pLDH	Parasite lactate dehydrogenase
Rf	Ratio of the distance migrated by a substance compared with the solvent front

RI	Resistance index
rpm	Revolutions per minute
S.D.	Standard deviation
SI	Selectivity index
SPE	Solid phase extraction
TLC	Thin layer chromatography
TMS	Tetramethylsilane
µg	Microgram(s)
µl	Microlitre(s)
µl/L	Microlitre(s) per litre
µm	Micrometer
UV	Ultraviolet
WHO	World Health Organization
w/v	Weight per volume

## LIST OF FIGURES

### CHAPTER 1

**Figure 1.1.** A diagram showing the lifecycle of *Plasmodium falciparum*.

**Figure 1.2.** World map showing the situation of malaria.

**Figure 1.3.** Map showing the degree of poverty in the world.

**Figure 1.4.** Map of the endemic malaria areas within South Africa.

**Figure 1.5.** Annual malaria cases and deaths in South Africa (1970-2003).

**Figure 1.6.** Annual malaria cases in South Africa (1989-2004).

**Figure 1.7.** Chemical structures of quinolines.

**Figure 1.8.** Chemical structures of proguanil and cycloguanil.

**Figure 1.9.** Chemical structures of artemisinin and its derivatives.

### CHAPTER 2

**Figure 2.1.** A flow chart showing drug discovery process.

### CHAPTER 3

**Figure 3.1.** A map showing all the nine provinces of South Africa and the country of Lesotho, which is surrounded by the Republic of South Africa.

### CHAPTER 4

**Figure 4.1.** A diagram showing four fractions (H201 – H204) obtained according to their colour intensities from the cold H<sub>2</sub>O root extract of *D. anomala* from Lesotho.

**Figure 4.2.** A schematic diagram of the fractionation of the cold DCM root extract of *D. anomala* from Lesotho into 10%, 5% and 1% fractions using SPE.

**Figure 4.3.** A schematic representation of 1% fractions (fractions 31 - 35) that were separated from fraction 35.

**Figure 4.4a.** The HPLC chromatogram of fraction 31 showing peaks MF4 and MF5.

**Figure 4.4b.** The HPLC chromatogram of fraction 32 showing peak MF7.

**Figure 4.5.** A plate set up for the antiplasmodial assay.

**Figure 4.6.** A plate set up for the cytotoxicity assay.

## CHAPTER 5

**Figure 5.1a.** The HPLC chromatogram of the cold DCM root extract of *D. anomala* from Lesotho.

**Figure 5.1b.** The HPLC chromatogram of the hot DCM root extract of *D. anomala* from Lesotho.

**Figure 5.2a.** The HPLC chromatogram of the cold DCM root extract of *D. anomala* from Sterkspruit.

**Figure 5.2b.** The HPLC chromatogram of the hot DCM root extract of *D. anomala* from Sterkspruit .

**Figure 5.3.** The antiplasmodial dose response curves showing the DCM extracts of the leaves and twigs and the roots of *D. anomala* collected from four locations (Lesotho, Sterkspruit, Reitz and Limopo).

**Figure 5.4.** The cytotoxicity dose response curves showing the DCM extracts of the leaves and twigs and the roots of *D. anomala* collected from four locations (Lesotho, Sterkspruit, Reitz and Limpopo).

**Figure 5.5.** The antiplasmodial and cytotoxicity dose response curves of organic and water extracts of the roots of *D. anomala* collected from Lesotho.

**Figure 5.6.** The antiplasmodial dose response curves of H<sub>2</sub>O extracts of the roots of *D. anomala* collected from four locations (Lesotho, Sterkspruit, Reitz and Limpopo).

**Figure 5.7.** The cytotoxicity dose response curves of the hot water root extract (hotplate: 2 hrs) of *D. anomala* from Limpopo.

**Figure 5.8.** The antiplasmodial and cytotoxicity dose response curves of the cold water root extract and cold water root extract fractions of *D. anomala* from Lesotho.

## CHAPTER 6

**Figure 6.1a.** The dose response curves showing the antiplasmodial activity of 10% SPE fractions from the cold DCM root extract (batch I) of *D. anomala* from Lesotho.

**Figure 6.1b.** The dose response curves showing the antiplasmodial activity of 10% SPE fractions from the crude DCM root extract (batch II) of *D. anomala* from Lesotho.

**Figure 6.2a.** The HPLC chromatogram of the percolate from the cold DCM root extract (batch I) of *D. anomala* from Lesotho.

**Figure 6.2b.** The HPLC chromatogram of the percolate from the cold DCM root extract (batch II) of *D. anomala* from Lesotho.

**Figure 6.3a.** The HPLC chromatogram of fraction 30 from the cold DCM root extract (batch I) of *D. anomala* from Lesotho. Run at a gradient of 50% - 70% MeCN: H<sub>2</sub>O.

**Figure 6.3b.** The HPLC chromatogram of fraction 30 from the cold DCM root extract (batch II) of *D. anomala* from Lesotho. Run isocratically at 60% MeCN: H<sub>2</sub>O.

**Figure 6.3c.** The HPLC chromatogram of fraction 40 from the cold DCM root extract (batch I) of *D. anomala* from Lesotho. Run at a gradient of 50% - 70% MeCN: H<sub>2</sub>O.

**Figure 6.3d.** The HPLC chromatogram of fraction 40 from the crude DCM root extract (batch II) of *D. anomala* from Lesotho. Run isocratically at 60% MeCN: H<sub>2</sub>O.

**Figure 6.3e.** The HPLC chromatogram of fraction 50 from the cold DCM root extract (batch I) of *D. anomala* from Lesotho. Run at a gradient of 50% - 70% MeCN: H<sub>2</sub>O.

**Figure 6.3f.** The HPLC chromatogram of fraction 50 from the cold DCM root extract (batch II) of *D. anomala* from Lesotho. Run isocratically at 60% MeCN: H<sub>2</sub>O.

**Figure 6.4.** The dose response curves showing the antiplasmodial activity of 5% SPE fractions from the cold DCM root extract (batch I and II) of *D. anomala* from Lesotho.

**Figure 6.5.** The dose response curves showing the antiplasmodial activity of 1% SPE fractions from the cold DCM root extract (batch I) of *D. anomala* from Lesotho.

**Figure 6.6.** The dose response curves showing the antiplasmodial activity of 10% SPE fractions (fractions 30, 40 and 50) from the cold DCM root extract of *D. anomala* from Sterkspruit.

**Figure 6.7a.** The HPLC chromatogram of fraction 30 from the cold DCM root extract of *D. anomala* from Sterkspruit.

**Figure 6.7b.** The HPLC chromatogram of fraction 40 from the cold DCM root extract of *D. anomala* from Sterkspruit.

**Figure 6.7c.** The HPLC chromatogram of fraction 50 from the cold DCM root extract of *D. anomala* from Sterkspruit.

## CHAPTER 7

**Figure 7.1.** Separation scheme for obtaining compounds MF4 and MF7A from the most active fractions, fractions 40 and 50.

**Figure 7.2a.** Reverse phase semi-preparative HPLC for fraction 40 run at a gradient of 50% - 70% MeCN: H<sub>2</sub>O.

**Figure 7.2b.** Reverse phase semi-preparative HPLC for fraction 50 run at a gradient of 50% - 70% MeCN: H<sub>2</sub>O.

**Figure 7.3.** The UV spectra of MF4 (A) and MF7 (B).

**Figure 7.4a.** LC/MS of peak MF7.

**Figure 7.4b.** NMR of peak MF7 run in GlaxoSmithKline (Middlesex, UK).

**Figure 7.5a.** Reverse phase semi-preparative HPLC for fraction 40 run at an isocratic elution of 50% MeCN: H<sub>2</sub>O.

**Figure 7.5b.** Reverse phase semi-preparative HPLC for fraction 40 run at an isocratic elution of 60% MeCN: H<sub>2</sub>O.

**Figure 7.5c.** Reverse phase semi-preparative HPLC for fraction 40 run at an isocratic elution of 70% MeCN: H<sub>2</sub>O.

**Figure 7.5d.** Reverse phase semi-preparative HPLC for fraction 40 run at an isocratic elution of 60% MeCN: H<sub>2</sub>O. The chromatogram shows the separation of components in peak MF7.

**Figure 7.5e.** The UV spectra of MF7A, MF7B and MF7C.

**Figure 7.6.** The analytical HPLC chromatogram of compound MF4.

**Figure 7.7.** The analytical HPLC chromatogram of compound MF7A.

**Figure 7.8.** The UV spectra of compounds MF4 and MF7A.

**Figure 7.9a.** LC-MS of compound MF4.

**Figure 7.9b.** APCI of compound MF4.

**Figure 7.9c.** ESI-HRMS of compound MF4.

**Figure 7.10a.** LC-MS of compound MF7A.



**Figure 7.10b.** APCI of compound MF7A.

**Figure 7.10c.** ESI-HRMS of compound MF7A.

**Figure 7.11a.** LC-MS of peak MF7B.

**Figure 7.11b.** APCI of peak MF7B.

**Figure 7.12a.** LC-MS of peak MF7C.

**Figure 7.12b.** APCI of peak MF7C.

**Figure 7.13.**  $^1\text{H}$ -NMR spectrum of compound MF7A ( $\text{C}_{30}\text{H}_{28}\text{O}_6$ ).

**Figure 7.14.**  $^{13}\text{C}$ -NMR spectrum of compound MF7A ( $\text{C}_{30}\text{H}_{28}\text{O}_6$ ).

**Figure 7.15.** COSY spectrum of compound MF7A ( $\text{C}_{30}\text{H}_{28}\text{O}_6$ ).

**Figure 7.16.** HSQC spectrum of compound MF7A ( $\text{C}_{30}\text{H}_{28}\text{O}_6$ ).

**Figure 7.17.** Chemical structure of compound MF7A ( $\text{C}_{30}\text{H}_{28}\text{O}_6$ ) isolated from fractions 40 and 50.

**Figure 7.18.**  $^1\text{H}$ -NMR spectrum of compound MF4 ( $\text{C}_{30}\text{H}_{28}\text{O}_6$ ).

**Figure 7.19.**  $^{13}\text{C}$ -NMR spectrum of compound MF4 ( $\text{C}_{30}\text{H}_{28}\text{O}_6$ ).

**Figure 7.20.** COSY spectrum of compound MF4 ( $\text{C}_{30}\text{H}_{28}\text{O}_6$ ).

**Figure 7.21.** HMQC spectrum of compound MF4 ( $\text{C}_{30}\text{H}_{28}\text{O}_6$ ).

**Figure 7.22.** HSQC spectrum of compound MF4 (C<sub>30</sub>H<sub>28</sub>O<sub>6</sub>).

**Figure 7.23.** The dose-response curves of the antiplasmodial activity of MF4 and MF7A on D10, K1 and RSA11 strains of *P. falciparum*.

**Figure 7.24.** The dose-response curves of the cytotoxicity of MF4 and MF7A on CHO cells.

**Figure 7.25.** The cytotoxicity dose response curves of emetine, MF4 and MF7A. MF4 and MF7A were isolated from a new batch of fraction 40 and screened on a new batch of CHO cells, to confirm the cytotoxicity of the two compounds.

**Figure 7.26.** Sesquiterpene lactones isolated from the aerial parts of *D. anomala* Sond. ssp. *anomala*.

**Figure 7.27.** Sesquiterpene lactones isolated from the aerial parts of *D. anomala* Sond. ssp. *cirsioides* (Harv) Willd.

**Figure 7.28.** Sesquiterpene lactones isolated from the roots of *D. anomala* Sond. ssp. *cirsioides* (Harv) Willd.

**Figure 7.29.** The  $\alpha$ -methylene- $\gamma$ -lactone group.

## LIST OF TABLES

### CHAPTER 2

**Table 2.1.** Plants used traditionally in South Africa to treat malaria and fevers.

**Table 2.2.** Examples of traditional medicinal plants that have shown *in vitro* antiplasmodial activity against *P. falciparum* strains.

**Table 2.3.** Some of the isolated phytochemical constituents that has shown *in vitro* antiplasmodial activity against *P.falciparum* strains and *in vivo* activity against *P. berghei*.

### CHAPTER 3

**Table 3.1.** Vernacular names of *D.anomala*

### CHAPTER 4

**Table 4.1.** Cold and hot extractions of the leaves and twigs and the roots of *Dicoma anomala* collected from four locations (Lesotho, Sterkspruit, Limpopo and Reitz).

**Table 4.2.** The R<sub>f</sub> values of bands from fraction 31 ran in benzene: acetic acid mobile phase on an aluminium coated TLC plate.

### CHAPTER 5

**Table 5.1.** The antiplasmodial activity of the extracts of *D. anomala* on the CQS (D10) strain of *P. falciparum* and the cytotoxicity of the antiplasmodial active extracts on the CHO cells.

**Table 5.2.** The antiplasmodial activity and the cytotoxicity of the organic extracts of *D. anomala* roots from Lesotho on the D10 strain of *P. falciparum*.

**Table 5.3.** The antiplasmodial activity and cytotoxicity of the fractions of the cold H<sub>2</sub>O root extract of *D. anomala* from Lesotho on the D10 strain of *P. falciparum*.

**Table 5.4.** Extraction yields of extracts of the leaves and twigs and the roots of *D. anomala* collected from the four locations (Lesotho, Sterkspruit, Limpopo and Reitz).

**Table 5.5.** Extraction yields of organic extracts and fractions of the cold water root extract of the roots of *D. anomala* from Lesotho.

## CHAPTER 6

**Table 6.1.** Extraction yields of 10% fractions from batch I and batch II of the cold DCM root extract of *D. anomala* from Lesotho.

**Table 6.2.** Extraction yields of 1% and 5% fractions from batch I and batch II of cold DCM root extract of *D. anomala* from Lesotho.

**Table 6.3.** The *in vitro* antiplasmodial activity of 10% and 5% SPE fractions from batch I and II of the cold DCM root extract of *D. anomala* from Lesotho on CQS (D10) strain of *P. falciparum* and the *in vitro* cytotoxicity of fractions 40 and 50 on CHO cells.

**Table 6.4.** The R<sub>f</sub> values of bands from fraction 31 ran in benzene: acetic acid mobile phase on an aluminium coated TLC plate. The bands highlighted in blue were the most concentrated ones.

**Table 6.5.** The % yields and the antiplasmodial activity of 10% SPE fractions from the cold DCM root extract of *D. anomala* from Sterkspruit on the D10 strain of *P. falciparum*.

## CHAPTER 7

**Table 7.1.**  $^1\text{H}$  and  $^{13}\text{C}$ -NMR spectral data for compound MF7A.

**Table 7.2.** The *in vitro* antiplasmodial activity of compounds MF4 and MF7A on CQS (D10) and CQR (K1 and RSA11) strains of *P. falciparum* and their cytotoxicity on CHO cells.

## ABSTRACT

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The study investigates the antimalarial potential of a Southern African traditional medicinal plant, *Dicoma anomala* (Sond.). The plant is used to treat a variety of treatments including fevers. The extracts of the leaves and twigs have been reported to treat malaria and fever but the treatments with the roots are unrelated to malaria.

The plant was collected from four locations (Lesotho, Sterkspruit, Limpopo and Reitz). The cold and hot DCM root extracts ( $IC_{50} \leq 2.48 \mu\text{g/ml}$ ) and the cold DCM leaf and twig extracts ( $0.68 \pm 0.025 \mu\text{g/ml} \leq IC_{50} \leq 3.14 \pm 0.12 \mu\text{g/ml}$ ) showed *in vitro* antiplasmodial activity against the CQS strain (D10) of *P. falciparum*. Although the selectivity indexes of the extracts were low (ranging from 2 – 9), they showed that the activity of the leaf and twig extracts and the root extracts were selective to the malarial parasite and therefore the activity was not due to cytotoxicity. The cold and hot water root extracts did not show an interesting antiplasmodial activity ( $13.92 \pm 0.52 \mu\text{g/ml} \leq IC_{50} \leq 69.46 \pm 2.16 \mu\text{g/ml}$ ), while the hot water root extract of Limpopo which showed moderate activity ( $IC_{50} = 8.44 \pm 0.25 \mu\text{g/ml}$ ) and very low cytotoxic effects on CHO cells ( $87.06 \pm 6.70 \mu\text{g/ml}$ ). Two water extract fractions (cold extraction) from the roots collected in Lesotho showed antiplasmodial activity ( $4.85 \pm 0.18 \mu\text{g/ml} \leq IC_{50} \leq 7.48 \pm 0.07 \mu\text{g/ml}$ ) indicating a 3-fold increase in antiplasmodial activity. The fractions had low cytotoxic effects on CHO cells ( $59.60 \pm 2.20 \mu\text{g/ml} \leq IC_{50} \leq 78.73 \pm 1.99 \mu\text{g/ml}$ ).

Bioactivity-guided fractionation of the cold DCM root extract of *D. anomala* from Lesotho ( $1.55 \pm 0.07 \mu\text{g/ml} \leq IC_{50} \leq 2.48 \pm 0.005 \mu\text{g/ml}$ ) led to the isolation of two novel dimeric sesquiterpenes of the guaianolide-type, MF4 and MF7A. The two compounds both had molecular mass of  $[M + H]^+ = 485.1964$ , which was consistent with the molecular formulae  $C_{30}H_{28}H_6$ . The two compounds were therefore isomers since they eluted at different retention times on the HPLC. Compounds MF4 and MF7A were screened for *in vitro* antiplasmodial activity

against three strains of *Plasmodium falciparum*. Compound MF4 was highly active against the CQS strain, D10 ( $0.28 \pm 0.01 \mu\text{g/ml}$ ) and the CQR strain, RSA11 ( $0.28 \pm 0.075 \mu\text{g/ml}$ ). Compound MF7A was also highly active against the CQS strain, D10 ( $0.20 \pm 0.01 \mu\text{g/ml}$ ) but was less active against the CQR strain, RSA11 ( $0.40 \pm 0.07 \mu\text{g/ml}$ ). Both compounds showed lower activity against the CQR strain, K1 (MF4:  $0.94 \pm 0.34 \mu\text{g/ml}$ ; MF7A:  $0.78 \pm 0.20 \mu\text{g/ml}$ ) compared to the other two strains. Compound MF4 has shown to be the promising lead compound for the development of antimalarials with a selectivity index of 16 compared to compound MF7A which had the selectivity index of 2 on CHO cells.

The study has also shown that the roots of *D. anomala* contain compounds with molecular ion masses  $[M + H]^+$  of 487, 489, 491, 524, 526 and 528. The peaks with molecular masses of 487, 489, 491 as well as that of compound MF7A (485) differed from each other by 2 amu and similarly for the 524, 526 and 528 masses. This indicated the hydrogenation of the double bond/s. The UV spectra of the peaks with ion masses of 487 and 489 masses were similar to that of compound MF7A, strongly indicating that they have similar chemical structures to compound MF7A. The peaks with ion masses of 524, 526 and 528 also showed that they might have similar chemical structures.

The study has shown the presence of two novel dimeric sesquiterpene isomers which have antiplasmodial activity against chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum*. It has also shown that the roots of *Dicoma anomala* contain compounds with similar chemical structures, which are possible dimeric sesquiterpenes and confirmed the traditional use of *D. anomala* in the treatment of fever, some of which could be malarial fevers.

## RESEARCH SUMMARY

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The resistance of the malarial parasite, *P. falciparum* to existing antimalarial drugs is on the rise, making it difficult to control the disease. This has raised a need to develop new malaria remedies. In the fight against malaria, traditional medicine has been explored as the potential source of malaria remedies. In South Africa, about 80% of patients consult a traditional healer. This study investigates the antimalarial potential of a Southern African traditional medicine, *Dicoma anomala* (Sond.). The plant is used for the treatment of a number of conditions including fevers, and was collected in Lesotho, Limpopo, Reitz and Sterkspruit. The *D. anomala* from Lesotho was collected in two batches (batch I in December 1996 and batch II in October 2003) while the ones from Sterkspruit, Limpopo and Reitz and were collected in October 2003.

The crude extracts of the plant were evaluated for *in vitro* antiplasmodial activity against the chloroquine-sensitive strain, D10 of *P. falciparum*. The activity was measured using parasite lactate dehydrogenase assay and any test sample with an  $IC_{50}$  of  $\leq 10$   $\mu\text{g/ml}$  was considered active. The cold dichloromethane (DCM) leaf and twig extracts of *D. anomala* from Sterkspruit, Limpopo and Reitz showed activity ranging from 0.68  $\mu\text{g/ml}$  to 3.14  $\mu\text{g/ml}$  and cytotoxicity on chinese hamster ovarian (CHO) cells ranged from 3.85  $\mu\text{g/ml}$  to 31.12  $\mu\text{g/ml}$  with the selectivity index (SI) of 2.78 to 9.91. The SI was measured by the  $IC_{50}$  CHO cells/  $IC_{50}$  D10 strain. The cold DCM root extracts of *D. anomala* from Lesotho, Sterkspruit, Limpopo and Reitz showed antiplasmodial activity of 0.48  $\mu\text{g/ml}$  to 2.48  $\mu\text{g/ml}$  and the SI from 3.84 to 7.29. The hot DCM root extracts from the four locations exhibited antiplasmodial activity from 0.42  $\mu\text{g/ml}$  - 1.44  $\mu\text{g/ml}$  and the SI from 4.48 - 6.90. The SI showed that the crude extracts were more potent towards the parasites than to the cells. It was found that the high performance liquid chromatography (HPLC) profiles of the cold and hot DCM root extracts of *D. anomala* from Lesotho and Sterkspruit were similar, which would explain the similarity in antiplasmodial activity. The cold and hot water root extracts of *D.*



*anomala* from the four locations did not show antiplasmodial activity ( $IC_{50}$  from 13.92  $\mu\text{g/ml}$  to  $>100 \mu\text{g/ml}$ ), except for the hot water root extract from Limpopo ( $IC_{50} = 8.44 \mu\text{g/ml}$  and  $SI = 10.32$ ).

The DCM, ethylacetate and chloroform root extracts of *D. anomala* from Lesotho were active ( $IC_{50} = 1.18 \mu\text{g/ml} - 2.48 \mu\text{g/ml}$ ) against the D10 strain of *P. falciparum* but the hexane, petroleum spirit and methanol were not active ( $IC_{50} = 10.67 \mu\text{g/ml} - 39.30 \mu\text{g/ml}$ ). Bioactivity-guided fractionation using solid phase extraction method (SPE) was carried out on the cold DCM root extract of *D. anomala* from Lesotho, which showed antiplasmodial activity ( $IC_{50}$ ) ranging from 1.55  $\mu\text{g/ml}$  (batch II) to 2.48  $\mu\text{g/ml}$  (batch I). The DCM extracts from both batches were fractionated using 10% increments mobile phases of acetonitrile and water (MeCN:H<sub>2</sub>O) to obtain 13 fractions (percolate, H<sub>2</sub>O wash, fractions 10-100 and acetone wash). The most active fractions were fractions 30, 40 and 50 with antiplasmodial activities ranging from 0.46  $\mu\text{g/ml} - 0.72 \mu\text{g/ml}$ . No further work was done on fraction 30 since it was part of an ongoing research in the Division of Pharmacology at the University of Cape Town. The study, therefore, continued with fractions 40 and 50.

The HPLC profiles of fractions 40 and 50 indicated that they needed to be purified further since they contained a number of peaks. Further purification of these fractions was carried out by fractionating the cold DCM extract using SPE method. The compounds were eluted with 5% and then 1% increment mobile phases of MeCN:H<sub>2</sub>O in the range 35% - 55% (to expand fractions 40 and 50) and in the range 31% - 35% (to expand fraction 35), respectively. The fractionation did not result in pure samples or fractions with fewer peaks as shown by their HPLC profiles. Nevertheless, all the 5% fractions showed antiplasmodial activity against the D10 strain of *P. falciparum* ranging from 0.23 - 1.09  $\mu\text{g/ml}$ . The 1% fractions exhibited antiplasmodial activity ranging from 0.21  $\mu\text{g/ml} - 8.13 \mu\text{g/ml}$  against D10 strain and three chloroquine-resistant strains, K1, RSA11 and FAC8 of *P. falciparum*. Although further purification of the cold DCM

root extract by SPE fractionation did not result in pure samples or fractions with fewer peaks, the fractionation did not result in the loss of activity of the cold DCM root extract but the activity was enhanced in some fractions.

Two compounds, MF4 and MF7A were isolated from fractions 40 and 50. Compound MF4 and MF7A were both isolated from fraction 40 and only compound MF7A was isolated from fraction 50. The compounds were isolated by reverse-phase semi-preparative HPLC and purified on analytical HPLC. The antiplasmodial activity of the two compounds ranged from 0.20 µg/ml to 0.94 µg/ml against D10, K1 and RSA11 strains. The cytotoxicity of the two compounds was determined on CHO cells. Compound MF4 showed cytotoxicity of 4.41 µg/ml and compound MF7A of 0.45 µg/ml. Of the two compounds, compound MF4 was the favourable one with the SI of about 16, indicating that it is more potent towards the parasites than the cells. Compound MF7A showed a very low SI of 2.3. The two compounds showed a narrow safety margin, which may hamper their potential as lead antimalarial compounds. However, there is possibility of structural modifications that could result in derivatives that are less cytotoxic with high antiplasmodial activity.

Compounds MF4 and MF7A, both had a molecular mass  $[M + H]^+$  of 485.1964, consistent with the molecular formula of  $C_{30}H_{28}O_6$ , indicating that they are dimeric sesquiterpenes. The two compounds eluted at different retention times on the HPLC, suggesting that they are possible isomers. This was further supported by their ion mass fragmentation patterns that were similar. The chemical structure of compound MF7A was elucidated by Mass spectrometry and 1D and 2D-NMR spectroscopy and was shown to be a novel dimeric sesquiterpene. The chemical structure of compound MF4 could not be fully elucidated due to difficulty in carbon and hydrogen assignments. Since the two compounds have been shown to be isomers, this means that compound MF4 has a similar chemical structure to compound MF7A but the two compounds could differ by structural conformation.

The molecular ion masses  $[M + H]^+$  of 6 peaks (MF5: 524, MF6B: 526, MF6E: 528, MF7B: 487, MF7C: 489 and MF8: 491) from fraction 40 were obtained. The ion masses of peaks MF7B, MF7C and MF8 as well as compound MF7A (485) differed from each other by 2 amu and similarly for peaks MF5, MF6B and MF6E. This indicated the hydrogenation of the double bond/s. It was observed that the UV spectra of peaks with 487 and 489 masses were similar to that of compound MF7A, strongly indicating that they have similar chemical structures to compound MF7A. The peaks with ion masses of 524, 526 and 528 also showed that they might have similar chemical structures. The compounds producing these peaks could not be isolated due to problems encountered with their separation and purification. The compound producing peak MF5 was unstable since it changed from powder form to oily form minutes after being dried.

## CONFERENCE PRESENTATION and AWARD NOMINATION

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Part of this work was presented as an oral presentation at the following conference:

***International Conference on Promotion and Development of Botanicals  
with International Coordination: Exploring quality, safety, efficacy and  
regulations. Kolkata, India, February 2005***

### **Abstract:**

#### **Antiplasmodial Compounds Isolated from a Southern African Medicinal Plant**

Malefa Tselanyane, Motlalepula Matsabisa, William E. Campbell and Peter I. Folb

Malaria control and eradication is hindered by resistant strains of *Plasmodium falciparum* parasites to commonly used antimalarial drugs. In search for new antimalarial drugs, a southern African medicinal plant was evaluated. Bioassay guided fractionation of the crude extract ( $IC_{50} = 2.0 \mu\text{g/ml}$ ) was carried out with 10% increment mobile phases of acetonitrile and water using solid phase extraction to afford 13 fractions. The most active fractions were found to be in fractions 30%, 40% and 50%. HPLC was used to isolate compounds from fractions 40% and 50% resulting in the isolation of two compounds. Compound 1 showed antiplasmodial  $IC_{50}$  of  $0.28 \mu\text{g/ml}$  and compound 2  $IC_{50}$  of  $0.19 \mu\text{g/ml}$ .

**The presentation of the abstract was awarded the Dr. Manjusree Pal Memorial Award for the best presentation in oral session.**

- The research project was also nominated for the ***South African Women in Science Awards*** in the category "***African Women Scientist Fellowship***". *Mail & Guardian*, 06 August 2004.

## OVERVIEW OF THE THESIS

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The thesis has eight chapters preceded by a dedication, a declaration, acknowledgements, abbreviations, lists of tables and figures, an abstract, research summary, a conference presentation, table of contents.

**Chapter 1** reviews the literature on various aspects of malaria. The chapter also covers the scope and objectives of the study.

**Chapter 2** discusses traditional medicine and the role of ethnopharmacology in the treatment of malaria.

**Chapter 3** focuses on the ethnobotany and ethnopharmacology of a Southern African traditional medicine, *Dicoma anomala* (Sond.).

**Chapter 4** details the methods used in the study.

**Chapters 5, 6 and 7** present the results of the study. Each chapter has an introduction, brief details of the methods, discussion and conclusion.

The general discussion and conclusions are presented in **Chapter 8**. This chapter reviews objectives achieved, problems encountered, future work and research prospects that may arise from the study findings. It also summarizes essential conclusions from the study. A list of references is attached at the end of chapter 8 followed by appendices.

## TABLE OF CONTENTS

Dedication .....	i
Declaration .....	ii
Acknowledgements .....	iii
List of Abbreviations .....	v
List of figures and tables .....	viii
Abstract .....	xiv
Research Summary .....	xxi
Conference Presentation .....	xxv
Overview of the Thesis .....	xxvi
Table of Contents .....	xxvii

## CHAPTER 1

### INTRODUCTION AND LITERATURE REVIEW

1.1. Brief History of Malaria .....	2
1.2. <i>Plasmodium</i> species affecting humans .....	2
1.3. Life cycle of <i>Plasmodium falciparum</i> .....	3
1.3.1. The Sexual Stage of <i>P. falciparum</i> in the Mosquito vector .....	3
1.3.2. The Asexual Stage of <i>P. falciparum</i> in the Liver .....	5
1.3.3. The Asexual Stage of <i>P. falciparum</i> in the Erythrocytes .....	5
1.4. The Pathogenesis of Malaria .....	6
1.5. Global Situation of Malaria .....	8
1.6. The Economic Burden of Malaria .....	9
1.7. Malaria in South Africa .....	11
1.8. Antimalarial Drugs: Modes of Action and Mechanism of Drug Resistance .....	16
1.8.1. Quinolines .....	16
1.8.1.1. Quinine .....	17
1.8.1.2. Quinidine .....	17

1.8.1.3. Chloroquine	18
1.8.1.3.1. Mechanism of Action of Chloroquine	18
1.8.1.3.2. Mechanism of Resistance to Chloroquine	18
1.8.1.4. Amodiaquine	19
1.8.1.5. Primaquine	19
1.8.1.6. Mefloquine	19
1.8.1.7. Halofantrine	19
1.8.2. Antifolates	20
1.8.2.1. Mechanism of Antifolate Action	21
1.8.2.2. Mechanism of Resistance to Antifolates	21
1.8.2.3. Sulphadoxine-pyrimethamine	22
1.8.2.4. Chlorproguanil-dapsone	23
1.8.2.5. Atovaquone-proguanil	23
1.8.3. Artemisinin	24
1.8.3.1. Artemisinin-based Combination Therapy (ACT)	26
1.9. Eradication and Control of Malaria	27
1.9.1. Mass Administrations of Antimalarial drugs for Malaria Control	28
1.9.2. Search for new antimalarial drugs	29
1.9.3. Scope of the Project	29

## CHAPTER 2

### TRADITIONAL MEDICINE

2.1. Traditional Medicine and Traditional Healing	32
2.2. Collection of Medicinal Plants	35
2.3. Plant Parts Used in the Treatment	35
2.4. Preparation Methods for Medicinal Plants	36
2.5. Traditional Medicine: A Global Perspective	36
2.6. Traditional Medicine and Traditional Healing in South Africa	38
2.7. Medicinal Plants as Source of Drugs	40

2.8. Medicinal Plants as Source of Antimalarial Drugs	42
2.9. Safety of Medicinal Plants	46
2.10. Challenges of using Traditional Medicine	47
2.11. Drug Discovery and Drug Development	48
2.11.1. Drug discovery	48
2.11.2. Drug Development	49

## CHAPTER 3

### ***Dicoma anomala* (Sond.) subspecies *anomala***

3.1. <i>Dicoma anomala</i> (Sond.) subspecies <i>anomala</i>	52
3.1.1. Description of the plant	52
3.2. The collection of the plant	52
3.3. Ethnopharmacology of <i>Dicoma anomala</i> (Sond.)	54
3.4. Phytochemical information on <i>Dicoma anomala</i> (Sond.)	56

## CHAPTER 4

### **MATERIALS AND METHODS**

4.1. Plant Collection and Extraction	58
4.1.1. Plant collection, identification and documentation	58
4.1.2. Preparation of cold extractions of the plant material	58
4.1.3. Preparation of hot extractions of the plant material	59
4.1.4. Separation of the cold water extract of the Lesotho roots	61
4.2. Solid Phase Extraction	62
4.2.1. Preparation of crude DCM extract from Lesotho for SPE	62
4.2.2. Fractionation of the crude DCM extract from Lesotho	63
4.2.3. Approaches used to purify 1% fractions	65
4.2.3.1. Method 1: Fractions 31-35 generated from fraction 40	65
4.2.3.2. Method 2: Fractions 31-35 reloaded onto the SPE column	65



---

4.2.3.3. Method 4: Fraction 31 purified on TLC	66
4.2.4. SPE fractionation of the crude DCM extract from Sterkspruit	67
4.3. High Performance Liquid Chromatography (HPLC)	67
4.3.1. Analytical HPLC conditions	68
4.3.1.1. Collection of peaks from fractions 31 and 32	68
4.3.2. Preparative HPLC conditions	70
4.3.2.1. Collection of MF4 and MF7A from fractions 40 and 50	70
4.4. Separation of the components in peak MF7	71
4.4.1. Recrystallization	71
4.4.2. C16 amide column	71
4.4.3. Isocratic elution of fraction 40	71
4.5. Purification of Compounds MF4 and MF7A	72
4.6. <i>In vitro</i> Antiplasmodial Activity	72
4.6.1. Reagents	72
4.6.2. <i>In vitro</i> culture of <i>Plasmodium falciparum</i>	73
4.6.3. Parasite synchronization	74
4.6.4. Parasite lactate dehydrogenase (pLDH) Assay	74
4.6.5. Preparation of stock solutions for the Antiplasmodial Assay	74
4.6.6. Antiplasmodial Assay	75
4.7. <i>In vitro</i> Cytotoxicity Assay	77
4.7.1. Reagents	77
4.7.2. MTT Assay	77
4.7.3. Sub-culturing cells	78
4.7.4. Preparation of stock solutions for the cytotoxicity assay	78
4.7.5. Plating cells	79
4.7.6. Cytotoxicity Assay	80
4.7.7. Selectivity Index	81
4.7.8. Resistance Index	81
4.8. Spectroscopy for Compounds MF4 and MF7A	82

**CHAPTER 5****EVALUATION OF THE CRUDE EXTRACTS OF  
*Dicoma anomala* FOR ANTIPLASMODIAL ACTIVITY**

5.1. INTRODUCTION .....	84
5.2. METHODS .....	84
5.3. RESULTS AND DISCUSSION .....	84
5.4. CONCLUSION .....	102

**CHAPTER 6****BIOACTIVITY-GUIDED FRACTIONATION OF THE CRUDE  
DICHLOROMETHANE ROOT EXTRACT OF  
*Dicoma anomala***

6.1. INTRODUCTION .....	104
6.2. METHODS .....	104
6.3. RESULTS AND DISCUSSION .....	105
6.4. CONCLUSION .....	124

**CHAPTER 7****THE ISOLATION, PURIFICATION, CHARACTERIZATION  
AND ANTIPLASMODIAL ACTIVITY OF COMPOUNDS MF4  
and MF7A**

7.1. INTRODUCTION .....	127
7.2. METHODS .....	127
7.3. RESULTS AND DISCUSSION .....	128
7.3.1. The Isolation of Compounds MF4 and MF7A .....	128
7.3.2. Separation of components in peak MF7 .....	131
7.3.3. Purification of compounds MF4 and MF7A .....	135

7.3.4. Mass Spectrometry -----	137
7.3.5. Nuclear Magnetic Resonance (NMR) -----	143
7.3.5.1. NMR spectra of compound MF7A -----	143
7.3.5.1.1. <sup>1</sup> H-NMR spectrum -----	143
7.3.5.1.2. <sup>13</sup> C-NMR spectrum -----	144
7.3.5.1.3. Correlation spectroscopy (COSY) -----	145
7.3.5.1.4. 2D-NMR spectrum (HSQC) -----	146
7.3.5.1.5. Proton and carbon assignments for Compound MF7A -----	150
7.3.5.2. NMR spectra of compound MF4 -----	152
7.3.5.2.1. <sup>1</sup> H-NMR spectrum -----	152
7.3.5.2.2. <sup>13</sup> C-NMR spectrum -----	153
7.3.5.2.3. Correlation spectroscopy (COSY) -----	154
7.3.5.2.4. 2D-NMR spectrum (HMQC and HSQC) -----	155
7.3.6. Antiplasmodial activity and Cytotoxicity of Compounds MF4 and MF7A -	157
7.3.7. Compounds isolated from <i>Dicoma anomala</i> -----	161
7.3.8. The activities of sesquiterpene lactones -----	164
7.4. CONCLUSION -----	165

## CHAPTER 8

### DISCUSSION AND CONCLUSIONS

#### Objectives achieved, Problems encountered and Future work

8.1. Objectives Achieved -----	169
8.2. Problems Encountered -----	174
8.3. Future Work -----	175

REFERENCES -----	179
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LIST OF APPENDICES -----	209
--------------------------	-----

Appendix A -----	210
Appendix B -----	214

Appendix C	218
Appendix D	224
Appendix E	227
Appendix F	235

# ***CHAPTER 1***

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## **INTRODUCTION AND LITERATURE REVIEW**

## 1.1. Brief History of Malaria

Malaria was once thought to be caused by a stink from the marshes, hence the name mal aria (Latin for 'bad air'). The discoveries made by Robert Koch (1843-1910) and his associates established that microbes and not evil vapours caused diseases like anthrax, tuberculosis and cholera. The causative agent of malaria shifted from bacterium to the pigment in the blood (haemozoin). A breakthrough came in 1880 when a French scientist, Alphonse Laveran (1845-1922) made a discovery that the causative agent of malaria is a protozoan parasite not a bacterium, he named this parasite *Oscillaria malariae*. Later, Italian scientists named this protozoan parasite *Plasmodium malariae*. In 1897 Ronald Ross discovered that malaria is transmitted via mosquitoes [Sherman, 1998].

Parasitic protozoa are single celled organisms known to cause a variety of diseases in humans and domestic animals. The parasitic protozoan diseases are a major cause of mortality and morbidity worldwide [Johnston et al., 1999].

## 1.2. *Plasmodium* species affecting humans

Malaria affects monkeys, rodents, birds, reptiles and humans [Donnelly et al., 2002]. There are four species of *Plasmodium* that cause malaria in humans, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium falciparum*. Of the four *Plasmodium* species, *Plasmodium falciparum* is the most prevalent and most virulent. *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale* do not generally cause severe disease although they are often linked with anemia and low birth weights. All three species are distributed unevenly throughout the tropical regions [Mims et al., 2004].

*Plasmodium vivax* invades reticulocytes (young red blood cells) and is responsible for most of the malaria infections worldwide. It causes an estimated 70 - 80 million cases of malaria each year. It is also distributed in some temperate regions and is predominant in Asia, the Western Pacific and the

Americas [Mendis et al., 2001]. *P. vivax* does not occur in West Africa as it requires Duffy blood group antigens (Fy<sup>a</sup> and Fy<sup>b</sup>) to invade the red blood cells [Miller et al., 1976]. These antigens do not occur in the majority of the population in West Africa [Mendis et al., 2001]. *P. vivax* has liver forms, hypnozoites (dormant stages) which can cause relapses if left untreated [Cogswell, 1992].

*Plasmodium malariae* has a low prevalence even in areas with intense malaria transmission. It causes quartan malaria (a pattern of fever with a 72-hour periodicity), which is a re-emerging parasitic disease worldwide. It can persist in the blood for years if not eradicated completely [Mims et al., 2004].

*Plasmodium ovale* is rarely reported as a cause of morbidity in malaria endemic areas and is mainly confined to tropical Africa and to limited areas of several islands of the Western Pacific region areas [Faye et al., 1998]. *P. ovale* like *P. vivax* also forms hypnozoites and thus causes relapses. Complications due to *P. ovale* malaria are infrequent and paroxysms of high fever may be uncomfortable [Facer & Rouse, 1991].

*Plasmodium falciparum* causes more than 95% of deaths worldwide and may result in life-threatening complications which can be fatal. Malaria parasites are transmitted in the human host through the bite from the female *Anopheles* mosquito. All four *Plasmodium* species have similar life cycles.

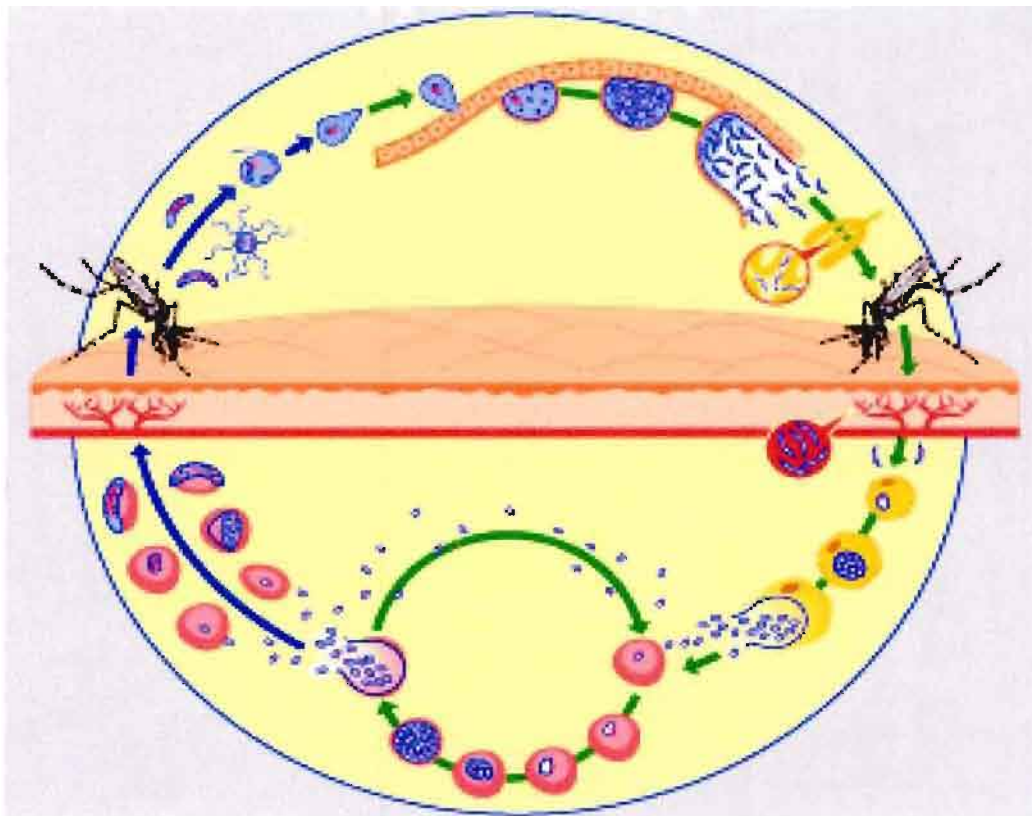
### **1.3. Life cycle of *Plasmodium falciparum***

The lifecycle of *Plasmodium species* is shown in figure 1.1.

#### **1.3.1. The Sexual Stage of *P. falciparum* in the Mosquito vector**

During the asexual cycle in the blood, a small percentage of the merozoites do not follow asexual multiplication, they develop into male and female gametocytes. These cells are only activated to produce gametes in the midgut of the mosquito

when ingested as part of the bloodmeal. The formation of gametes is followed by fertilization resulting into a diploid zygote. The zygote undergoes meiotic division and develops into a motile ookinete. The ookinete penetrates the cells of the midgut wall of the mosquito to form an oocyst on the basal lamina. The oocysts are located between the epithelium and the basal lamina of the midgut. Mature oocysts burst to release sporozoites into the haemolymph. This process happens approximately 17 days after the blood meal. From the haemolymph, the sporozoites invade the salivary glands. They then move into the secretory cavity where they remain viable until they are transmitted to the host during the mosquito's bloodmeal [Mims et al., 2004].



**Figure 1.1.** A diagram showing the lifecycle of *Plasmodium falciparum*.

Reproduced with permission from TDR/Wellcome Trust.

(<http://www.who.int/tdr/diseases/malaria/lifecycle.htm>)



### **1.3.2. The Asexual Stage of *P. falciparum* in the Liver**

Parasites are brought into the human bloodstream through the bite of the female *Anopheles* mosquito. During the bloodfeed, the mosquito probes the skin of the host and release sporozoites, which remain in the skin for more than 5 minutes [Sidjanski & Vanderberg, 1997]. Upon entering the bloodstream, sporozoites are quickly transported to the liver where they invade hepatocytes. This is where the sporozoites multiply, mature into schizonts and rupture to produce 10 000 - 40 000 intrahepatocellular merozoites. Despite this huge number of parasites generated, the plasmodial infection of the liver is asymptomatic [Mims et al., 2004].

### **1.3.3. The Asexual Stage of *P. falciparum* in the Erythrocytes**

Merozoites move from the liver to invade erythrocytes (red blood cells) in the peripheral circulation approximately a week after infection. Infection of erythrocytes by *P. falciparum* is initiated when the extracellular merozoite stage enters the red blood cell to form an intracellular ring. The invasion process takes approximately 30 seconds with the attachment of the apical end of the merozoite to the membrane of the erythrocyte to form a tight junction. This process is mediated by specific receptor-ligand interactions between parasite-encoded proteins and erythrocyte surface receptors. This is followed by the invagination of the erythrocyte membrane around the merozoite to form a parasitophorous vacuole [Dvorak et al., 1975; Aikawa et al., 1978]. The ring forms of the parasite mature into trophozoites, which develop into schizonts and mature to form merozoites which are released back into the circulation.

After invading erythrocytes, the merozoites multiply to produce up to 32 new merozoites, which then re-infect red blood cells every 48 hours. Within the erythrocyte, the parasite survives by feeding on the globin component of haemoglobin, which is the source of amino acids for the parasite. The haem is toxic to the parasite, however, the parasite has developed haem detoxification

mechanisms whereby haem is polymerised to form haemozoin, a dark pigment within the food vacuole. Haem polymerisation is a nonenzymatic process which requires preformed haemozoin, histidine-rich proteins and lipids [Howard et al., 1986; Sullivan et al., 1996; Fitch et al., 1999; Pandey et al., 2003].

#### 1.4. The Pathogenesis of Malaria

The initial symptoms of malaria infection such as fever, chills, nausea, vomiting, headache, abdominal and back pain appear approximately on day 11 after infection by the parasite [Berman, 2004]. *P. falciparum* can parasitize a large number of erythrocytes if untreated by chemotherapy and uncontrolled by the host immunity because the parasite has evolved mechanisms of evading the host immune response. *P. falciparum* has surface proteins called *P. falciparum* erythrocyte membrane protein-1 (PfEMP1) which act by blocking acquired immune response to infection and mediate adhesion of infected erythrocytes to the microvascular endothelium thus avoiding host clearance in the spleen [Langreth and Peterson, 1985]. This result in severe malaria, organ dysfunction, coma and death since vital organs such as the brain, liver, lungs, spleen, intestine and kidneys are affected [Warrel, 1987].

Severe malaria is associated with hyperparasitaemia (> 5%), severe anaemia caused by the destruction of red blood cells, cerebral malaria caused by the obstruction of small vessels of the brain by sequestered parasites, metabolic acidosis leading to metabolic distress, hypoglycaemia, hypopyrexia (>39.8 °C) [WHO, 2000].

The severity of malaria is believed to be due to the ability of *P. falciparum* infected red blood cells to adhere in the small blood vessels of major organs. Several cell adhesion molecules that are involved in this process include intercellular adhesion molecule-1 (ICAM-1), CD36, CD31 and P-selectin [Berendt et al., 1989; Oquendo et al., 1989; Treutiger et al., 1997; Udomsangpetch, 1997]. The transmission and survival of the malarial parasite depends on the variable

adhesion molecules that are displayed at the surface of *P. falciparum* infected erythrocyte. The most prominent of these variable antigens is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) [Hayward et al., 1999].

Some people with genetic traits that alter the red blood cell structure are protected against malaria. Populations in sub-Saharan Africa, parts of the Middle East and India that carry the sickle-cell genetic trait are partially protected against severe malaria. Sickle-cell trait is an inheritance of two abnormal allelomorphic genes that controls the formation of  $\beta$ -globin [Cook, 1996]. Thalassemia, a disorder of hemoglobin synthesis, renders the red blood cell less favourable for the development of malarial parasites. People suffering from thalassemia are therefore protected against severe malaria. There is a high frequency of thalassemia in populations in parts of Southeast Asia, Africa and the Mediterranean region [Cook, 1996].

Glucose-6-phosphate dehydrogenase (G6PD) deficiency also protects against severe malaria. In red cells, Glucose-6-phosphate dehydrogenase in the hexose monophosphate pathway is a source of NADPH (nucleotide adenosine diphosphate hydrogen). Deficiency of G6PD exposes the red blood cell to oxidation of haemoglobin making it difficult for parasite survival. Populations in sub-Saharan Africa, Saudi Arabia and Southeast Asia have an advantage against malaria because of high frequency of G6PD deficiency [Cook, 1996].

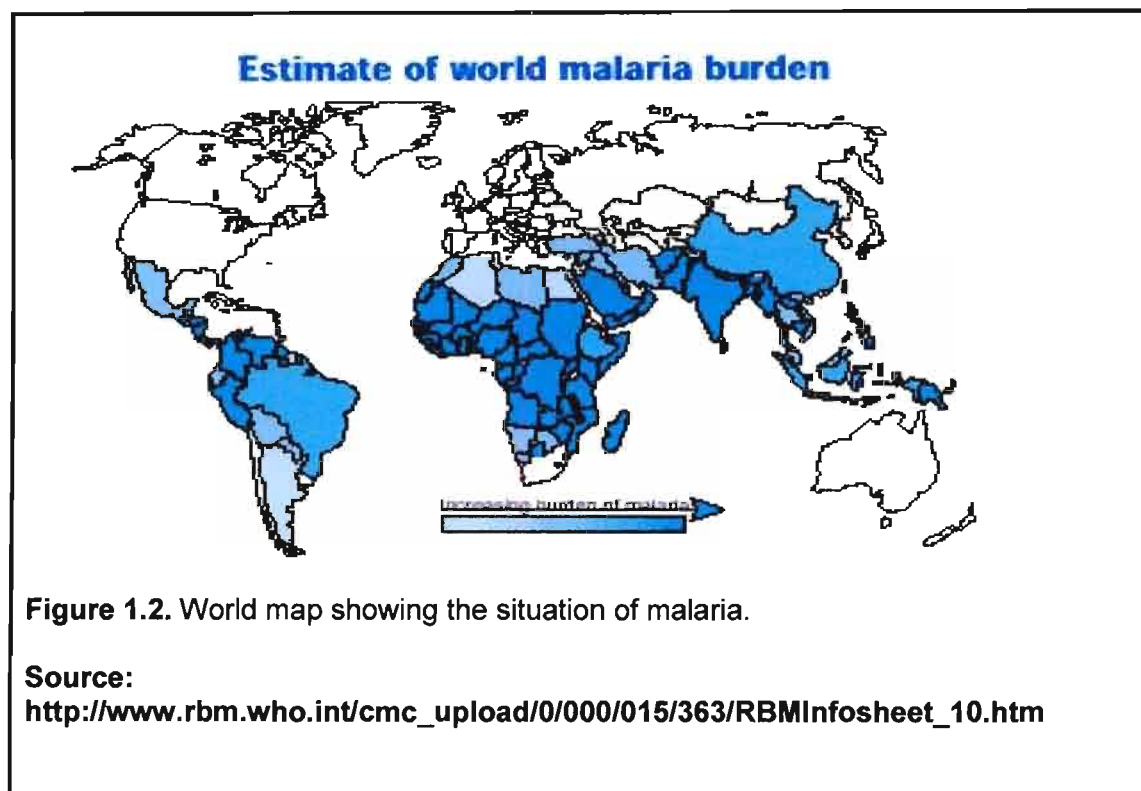
*Plasmodium vivax* merozoites have a Duffy binding protein which requires a Duffy receptor on erythrocytes for invasion of erythrocytes by the merozoites [Miller et al., 1976]. *Plasmodium vivax* infections are extremely rare in West Africa where the population has a Duffy-negative trait and are therefore completely protected against vivax malaria [Mendis et al., 2001].

## 1.5. Global Situation of Malaria

Malaria continues to be a life-threatening infectious disease, which is a public health problem worldwide and it affects the tropical and sub-tropical regions of the world. Malaria does not only affect people residing in these regions, travelers to the malaria endemic regions are at risk of being infected as well [Steffen et al., 1987; Jelinek et al., 2002].

An estimated 300 - 500 million clinical cases of malaria occur annually throughout the world with a mortality of 2 - 3 million deaths per year [WHO, 1997]. About 90% of these cases and 90% of all malaria deaths occur in Africa, in particular sub-Saharan Africa (figure 1.2). Over 75% of these deaths affect children younger than 5 years in tropical Africa. The actual figures could be higher owing to under-reporting and difficulties in diagnosis. Malaria death toll is estimated to double in the next 20 years if no new control measures are developed [Bremar, 2001]. Falciparum malaria infection in Africa tends to be endemic with high transmission rates while in South America, the Indian subcontinent, Southeast Asia and China, transmission tends to be lower and seasonal in nature [Winstanley, 2000].

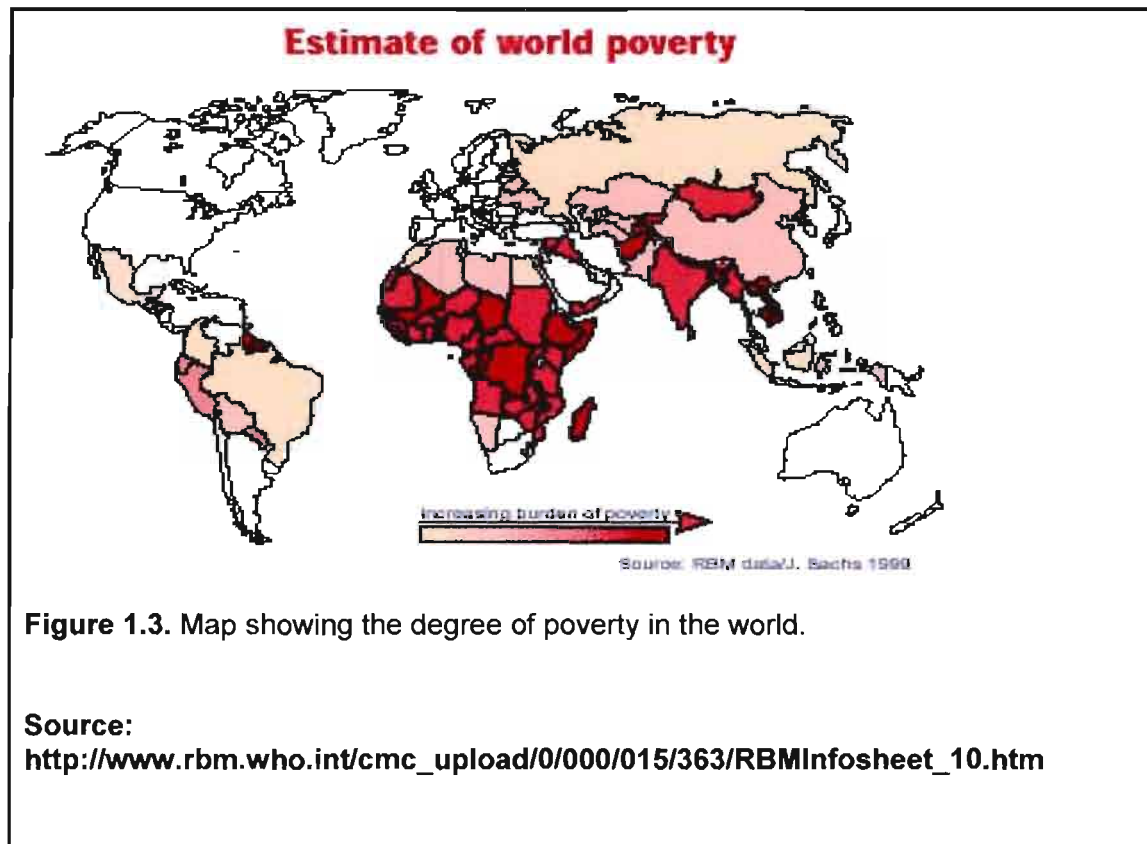
Pregnant women infected with malaria are also at risk. The risks include maternal anaemia, intrauterine growth retardation, congenital malaria infection and low birth weight. The low birth weight is the main cause of infant mortality. Infant deaths due to malaria infection in pregnancy are estimated at 75 000 - 200 000 [Bloland et al., 1996; Parise et al., 1998; Steketee et al., 2001].



## 1.6. The Economic Burden of Malaria

Malaria does not only affect the health of the nations, it affects its wealth as well with significant direct and indirect costs. Individual or family direct costs include spending on insecticide treated mosquito nets, doctors' fees, antimalarial drugs, transport to health facilities, accompanying family members and support during hospital stays. Public direct costs include spending by government on maintaining health facilities and health care infrastructure, vector control, research and education. The indirect costs on the other hand include lost productivity or income associated with illness or death. This results from the cost of lost workdays and absenteeism from work. The future of the children is also affected since their education is hampered by absenteeism in schools and permanent damage due to severe episodes of malaria [Roll Back Malaria Infosheet].

Recently it has been shown that malaria is a major constraint to economic development. There is a correlation between malaria and poverty since poverty is mostly concentrated in the tropical and subtropical regions where the transmission of malaria is high (figure 1.3).



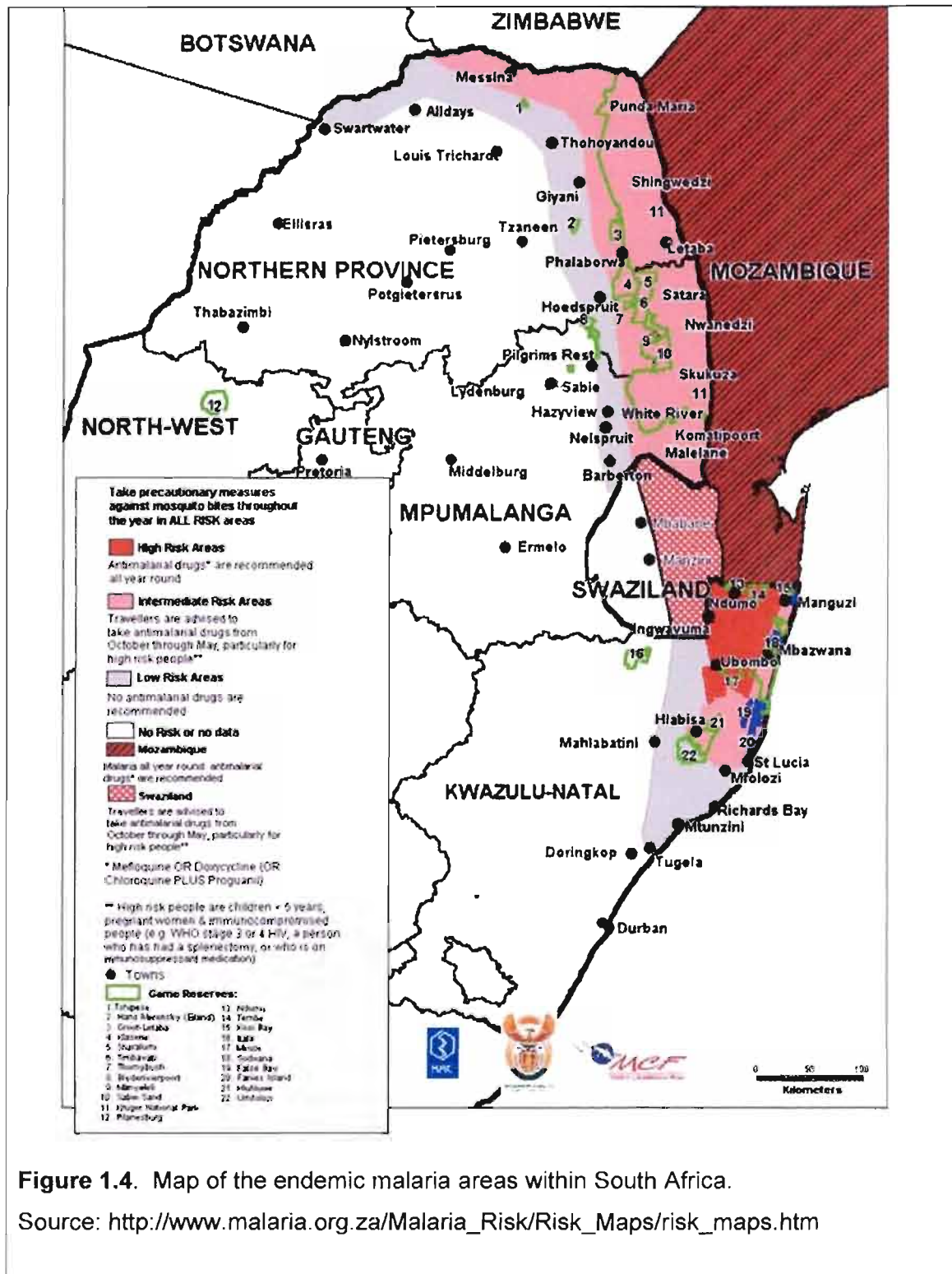
Africa is the poorest continent with most of its countries affected by malaria. The only parts of Africa free of malaria are the northern and southern extremes, which have the richest countries in the continent [Gallup and Sachs, 2001]. The economic progress in developing countries affected with malaria is hampered because there is less foreign and local investment in such countries. The trade and tourism industries bring in money to the country but are affected as travelers are reluctant to go to malaria endemic countries [Gallup and Sachs, 2001; Roll Back Malaria Infosheet].

Growth of income per capita from 1965 – 1990 for countries with severe malaria has been 0.4% per year compared to 2.3% per year for other countries. Of the countries with severe malaria, more than a third had negative growth. Data from countries that have eliminated malaria have shown that they have had an accelerating economic growth [Gallup and Sachs, 2001].

Climate and ecology are the main determinants of the severity of malaria. This is supported by the fact that wealthy countries still battle with controlling malaria because of their geographical location. Oman, which owes its wealth to oil has a growth income per capita of almost \$10 000 but there is severe malaria throughout the country. However, the remote areas of the desert and high altitude are not affected. Another country is the United Arab Emirates, which has the highest growth income in the world but is battling with malaria control [Gallup and Sachs, 2001]. Although it is acceptable that malaria is linked to poverty, there are also additional factors such as the geographical location, economic policies and other major tropical diseases.

## **1.7. Malaria in South Africa**

Malaria transmission in South Africa is seasonal due to local climate. The malaria season is experienced between October and May with peak transmission between January and February. Mpumalanga, Limpopo (formerly Northern Province) and KwaZulu-Natal are three provinces in South Africa which are malaria endemic areas (figure 1.4). The North West and the Northern Cape Provinces occasionally experience limited malaria transmission [DoH, 2000; DoH National Malaria Update, 2003].

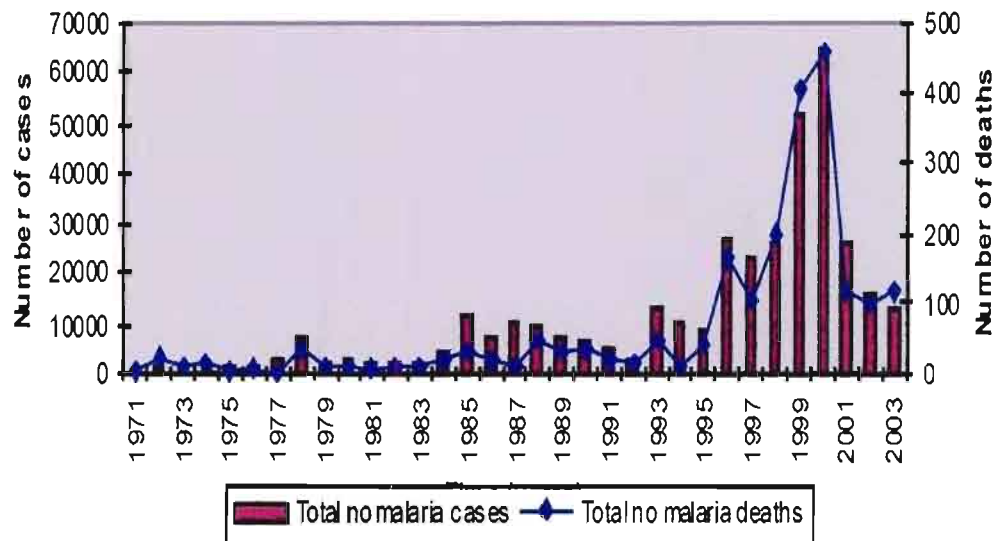


**Figure 1.4.** Map of the endemic malaria areas within South Africa.

Source: [http://www.malaria.org.za/Malaria\\_Risk/Risk\\_Maps/risk\\_maps.htm](http://www.malaria.org.za/Malaria_Risk/Risk_Maps/risk_maps.htm)



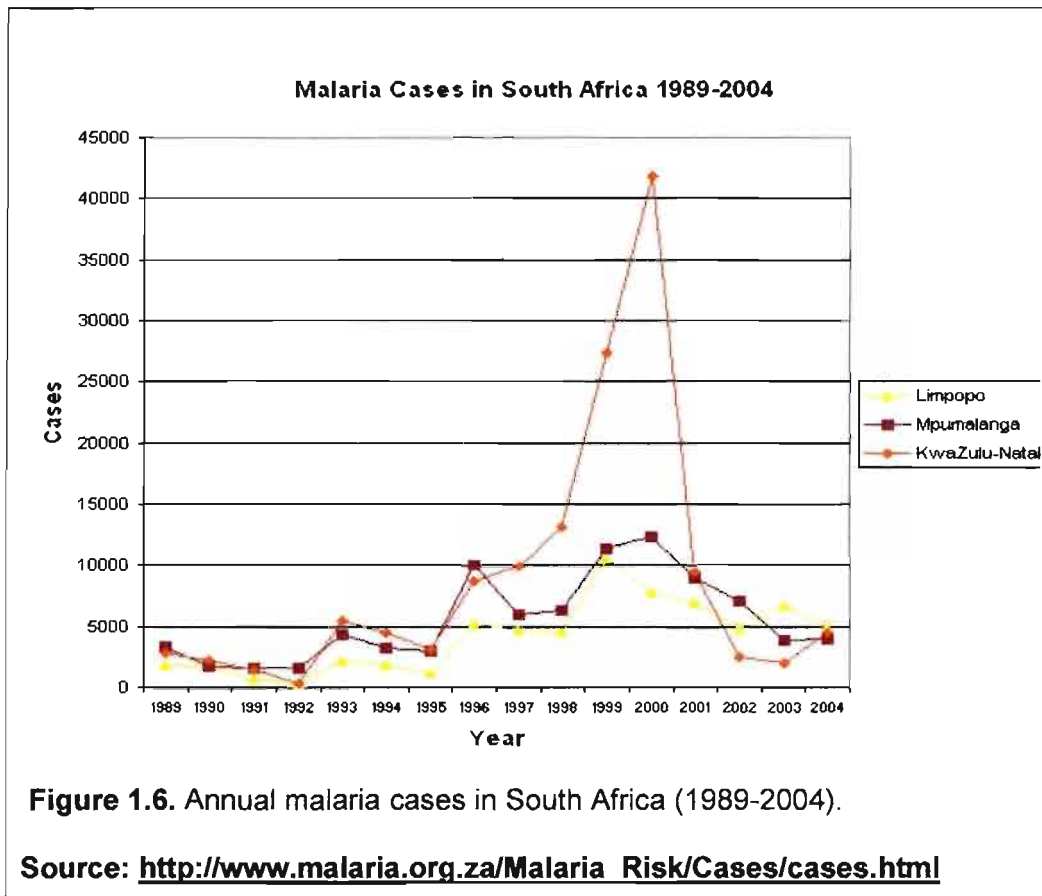
South Africa had a significant increase of malaria cases and deaths since 1996 and experienced malaria epidemic in 2000 followed by a decrease in the number of malaria cases in 2001 (figure 1.5). Among all the provinces, KwaZulu-Natal had the highest number of malaria cases (figure 1.6).



**Figure 1.5.** Annual malaria cases and deaths in South Africa (1970-2003).

**Source:** Department of Health, National Malaria Update, December 2003

<http://www.doh.gov.za/facts/index.html>



Malaria in KwaZulu-Natal is hypoendemic, meaning that the transmission intensity and incidence of malaria are sufficiently low that the population has little or no immunity. Transmission occurs during the rainy season (January to June) with peak transmission in April. Chloroquine was replaced as the first-line drug with sulfadoxine-pyrimethamine in KwaZulu-Natal in 1998 due to chloroquine-resistant parasites [Roper et al., 2003]. Sulfadoxine-Pyrimethamine started losing its effectiveness during 1995 - 1999 period in KwaZulu-Natal. The number of patients who did not clear the parasites within 14 days of treatment increased to 70% in 2000 [Bredenkamp et al., 2001] causing the province to switch to co-artemether in 2001. The introduction of artemisinin-based combinatorial therapy in KwaZulu-Natal, resulted in over 75% reduction in malaria cases and deaths within one year [Malaria Research Programme; DoH, 2001].

Limpopo Province borders on Zimbabwe in the north and Mozambique in the east. It is a high risk area for malaria during the summer months of November to May. People in the north-eastern region are mostly affected with 85% of the cases being due to *P. falciparum* infections [Voetseder et al., 2004].

Mpumalanga is bordered by Mozambique and Swaziland in the east. The high seasonal malaria transmission peaks between October and April. In 1997 Limpopo and Mpumalanga Provinces changed treatment from chloroquine to sulfadoxine-pyrimethamine as the first line treatment [Malaria Research Programme].

In October 1999, the Lubombo Spatial Development Initiative programme, which comprises of South Africa, Mozambique and Swaziland was launched [DoH Report, 2001/2002; Malaria Research Programme]. The objective of this initiative was to control malaria in Maputo Province, Mozambique and to reduce malaria in the border areas of South Africa and Swaziland. The eradication of malaria in these areas will contribute towards the economy development through tourism as well as creating sustainable employment. Reduction in malaria cases has been observed since the inception of this programme. A 90% reduction in Swaziland, an 88% reduction in Maputo Province, Mozambique, a 90% reduction in KwaZulu-Natal and a 75% reduction in Mpumalanga, South Africa [Malaria Research Programme].

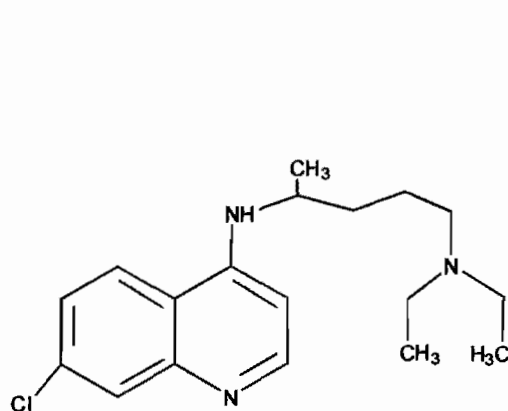
The change of the first-line treatment to co-artemether in some areas, the re-introduction of DDT spraying and the regional approach to malaria control in the Lubombo Spatial Development Initiative are thought to be partly the reasons in the reduction of malaria cases. The World Health Organization awarded the South African Health Department a prize for the best managed malaria control programme on the African continent, owing to the sharp decrease in malaria morbidity and mortality [WHO-OMS; Malaria Research Programme].

## 1.8. Antimalarial Drugs: Modes of Action and Mechanism of Drug Resistance

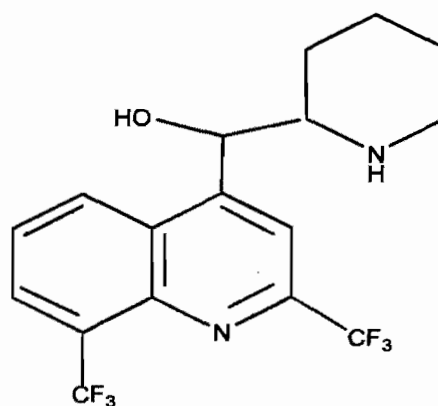
Drug resistance is the ability of the malaria parasite to survive and multiply in the presence of drug levels that normally destroy or prevent the multiplication of parasites [Bruce-Chwatt et al., 1981].

### 1.8.1. Quinolines

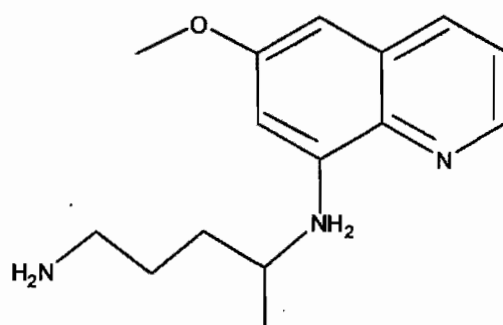
The chemical structures of some of the quinolines are shown in figure 1.7.



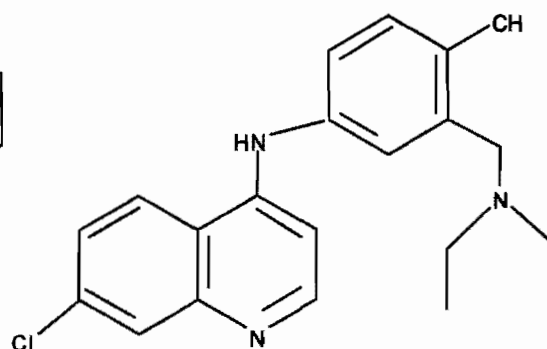
Chloroquine



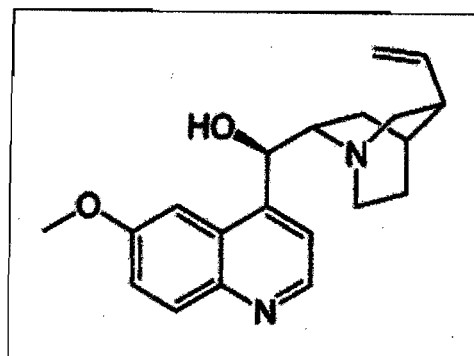
Mefloquine



Primaquine



Amodiaquine



Quinine

**Figure 1.7.** Chemical structures of quinolines.

#### 1.8.1.1. Quinine

Quinine was the first antimalarial drug obtained from the bark of *Cinchona* spp. which is native to South America. This alkaloid was first isolated in 1820 by French chemists, Joseph Pelletier and Jean Biename Caventou [Foley and Tilley, 1998]. Quinine is still used today for severe cases of chloroquine-resistant malaria in Africa. However, it is used as a second or third line drug in Africa but less so in Southeast Asia due to widespread resistance in the region [Winstanley, 2001, Wongsrichanalai et al., 2002]. Treatment with quinine is not well tolerated due to being extremely bitter. Quinine has maximum effects on late rings, trophozoites and early schizonts [Skinner, 1996]. It is also gametocidal for *P. falciparum* and *P. vivax*. It acts by inhibiting the formation of the malaria pigment, haemozoin.

#### 1.8.1.2. Quinidine

Quinidine is also an alkaloid obtained from the bark of *Cinchona* spp. or it can be prepared from quinine. Like quinine, it has schizontocidal effect and is gametocidal for *P. falciparum* and *P. vivax* but is more active than quinine for *P. falciparum*. Quinidine is not used as an antimalarial in most African countries, it is mainly used as such in the US. It is also used as an antiarrhythmic drug.

### **1.8.1.3. Chloroquine**

Chloroquine is a synthetic antimalarial of 4-aminoquinoline derivatives. It is a blood schizonticidal agent and has gametocidal effects on *P. malariae* and *P. vivax* [AHFS Drug Information, 2000]. Chloroquine was first introduced as an antimalarial drug in 1945 due to its potency and low cost [Foley and Tilley, 1998].

#### **1.8.1.3.1. Mechanism of Action of Chloroquine**

The mechanism of action of chloroquine is uncertain but it is widely acceptable that chloroquine acts by inhibiting haemozoin formation in the food vacuole of the parasite [Fitch et al., 1982]. Free heme is toxic to the parasites, hence the parasite has developed a mechanism of heme detoxification by polymerizing heme to form haemozoin. Chloroquine accumulates in the parasite digestive vacuole by pH gradient and kills trophozoites by forming a complex with heme thereby inhibiting heme detoxification [Fitch et al., 1982; Dorn et al., 1998].

#### **1.8.1.3.2. Mechanism of Resistance to Chloroquine**

Resistance of *P. falciparum* to chloroquine developed slowly over the years. It was first observed in 1950s – 1960s in Asia and Americas and in the 1970s in Africa [Su et al., 1997]. Chloroquine has widespread resistance in Southeast Asia and in most parts of Africa but it is still used for economic reasons even though its efficacy is on the decline [Winstanley, 2001; Trape, 2001].

Accumulation of chloroquine in the vacuole is diminished in chloroquine-resistant strains. Opinion is divided on the mechanisms involved in accumulation of less chloroquine in chloroquine-resistant parasites. Resistance reversal of chloroquine by drugs such as verapamil, desipramine and chlorpromazine suggests that an enhanced chloroquine efflux by a multidrug resistant mechanism may be implicated [Reed et al., 2000]. Resistance of *P. falciparum* to chloroquine has been linked to mutations in the *P. falciparum* multidrug resistance (*pfmdr1*) gene

and to the *P. falciparum* chloroquine related transporter (pfcr1) gene [Foote et al., 1990; Sidhu et al., 2002].

#### **1.8.1.4. Amodiaquine**

Amodiaquine is a chloroquine analogue used since the 1940s [Ngouesse et al., 2001]. Amodiaquine was discontinued in the 1980s due to its prophylactic use resulting in neutropenia and hepatitis [Hatton et al., 1986]. The prevalence of chloroquine-resistant strains has resulted in the reinvestigation of the prophylactic use of amodiaquine in regions of chloroquine resistance. Amodiaquine, like chloroquine, inhibits haemozoin formation.

#### **1.8.1.5. Primaquine**

Primaquine is a synthetic 8-aminoquinoline derivative that was introduced in the early 1950s for the treatment of liver hypnozoites in *P. vivax* and *P. ovale* infections. In Glucose-6-phosphate dehydrogenase deficient persons, primaquine can cause fatal haemolysis. It acts by interfering with the DNA of the parasite [AHFS Drug Information, 2000].

#### **1.8.1.6. Mefloquine**

Resistance of chloroquine during the 1960s – 1970s in the Americas and Southeast Asia led to the development of mefloquine. Mefloquine, a fluorinated 4-quinolinemethanol derivative, is a substituted analogue of quinine which is active against schizont stages of the parasite. It has similar mode of action to that of chloroquine and quinine. Mefloquine is well tolerated but expensive. Resistance to mefloquine has been linked with the *pfmdr*-N86Y mutation [May and Meyer, 2003].

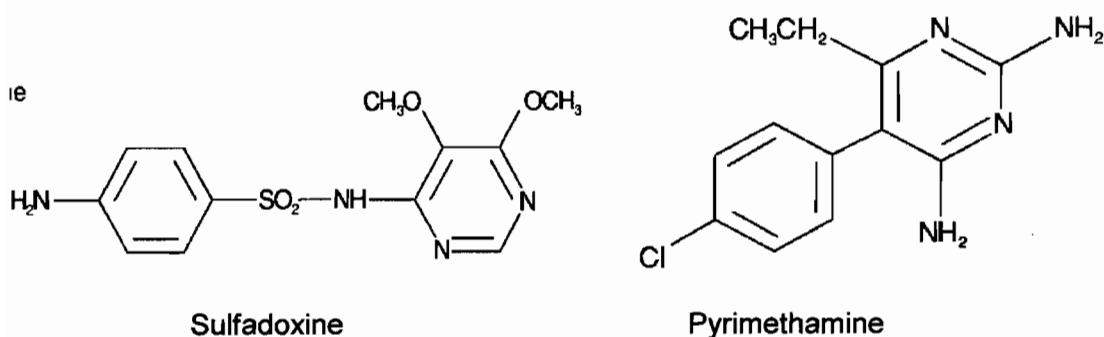
#### **1.8.1.7. Halofantrine**

Halofantrine was also introduced in the 1960s - 1970s due to chloroquine resistance. Halofantrine is better tolerated than mefloquine [White, 1996]. The

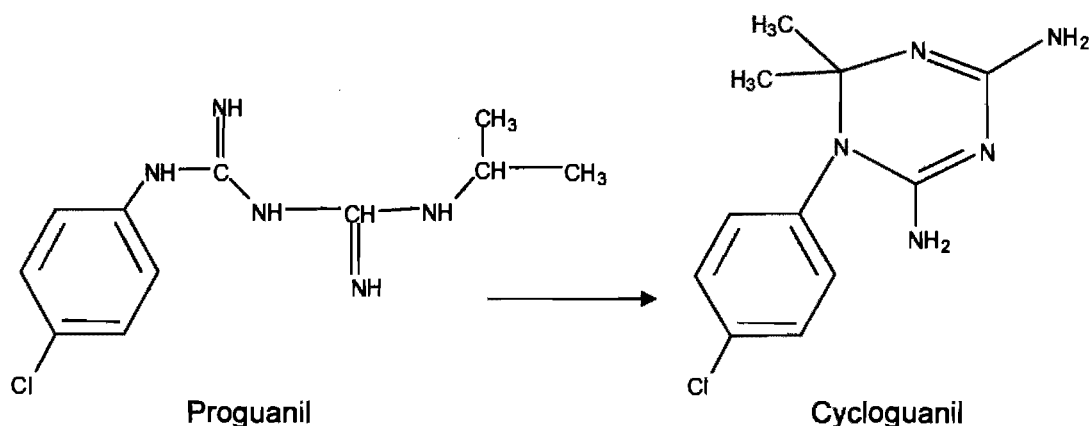
adverse effect of halofantrine is cardiotoxicity. As with mefloquine, halofantrine is expensive and the resistance to this drug has also been linked to the *pfmdr*-N86Y mutation.

### 1.8.2. Antifolates

The antifolate drugs (pyrimethamine, proguanil, cycloguanil, sulfadoxine) interact with the folate synthesis pathway by inhibiting the synthesis of folic acid, which is the essential precursor in the *de novo* synthesis of pyrimidines needed for DNA replication and transcription [AHFS Drug Information, 2000]. The two enzymes, dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) are involved in the folate synthesis pathway of *Plasmodium falciparum*. Malarial parasites are unable to take up pyrimidines as well as derivatives of folate from the host, therefore in the presence of antifolate drugs the two enzymes are inhibited resulting in the inhibition of pyrimidine synthesis thereby killing the parasite. Figure 1.8 below shows the chemical structures of antifolate drugs used for the treatment of malaria.







**Figure 1.8.** Chemical structures of proguanil and cycloguanil. Cycloguanil is derived *in vivo* from the pro-drug, proguanil.

#### 1.8.2.1. Mechanism of Antifolate Action

Proguanil acts by inhibiting pyrimidine biosynthesis by blocking DHFR through its active metabolite cycloguanil, therefore leading to the inhibition of nucleic acid synthesis. Pyrimethamine binds with high affinity to the enzyme, DHFR thereby inhibiting the activity of the enzyme [Hyde, 1990]. Sulfadoxine acts on DHPS by competing with the enzyme substrate, *p*-aminobenzoic acid [Wang et al., 1997].

#### 1.8.2.2. Mechanism of Resistance to Antifolates

Resistance of *P. falciparum* to pyrimethamine has been linked to point mutations in the *dihydrofolate reductase* (*dhfr*) gene at codon 108 (serine→asparagine), at codon 51 (asparagine→isoleucine) and at codon 59 (cysteine→arginine).

Resistance to sulfadoxine has been linked to mutations in the *dihydropteroate synthase* (*dhps*) gene at codon 437 (alanine→glycine), at codon 540 (glutamic acid→lysine), at codon 436 (serine→alanine or phenylalanine), at codon 581 (alanine→glycine) and at codon 613 (alanine→threonine/serine) [Brooks et al., 1994, Khalil et al., 2002; Kublin et al., 2002].

Due to increasing resistance to antifolate drugs and other antimalarial drugs, combination therapy was introduced. An effective combination therapy will consist of a drug with a short half-life and one with a long half-life. The short acting drug eliminates parasites rapidly and the long acting one will ensure that the remaining parasites are also eliminated.

#### **1.8.2.3. Sulfadoxine-pyrimethamine**

Sulfadoxine-pyrimethamine, also known by the brand name Fansidar®, is a fixed combination of two antifolate drugs, sulfadoxine and pyrimethamine used for the treatment of uncomplicated falciparum malaria. Fansidar® is an affordable and accessible drug that was used until recently in malaria endemic countries for the treatment of chloroquine-resistant strains. It was introduced in the 1980s and has until recently become the first-line drug in many countries. Eight African countries (Malawi, Kenya, Botswana, Zambia, Ethiopia, Tanzania, Zimbabwe and South Africa) switched to Fansidar® as the first-line treatment drug against uncomplicated malaria but the drug is expected to be ineffective by 2006 - 2007 since failure rates of 20% - 45% and 60% in separate trials were observed [Winstanley, 2001; Winstanley et al., 2002]. In the middle 1980s Fansidar was discontinued in the United States as its prophylactic use resulted in erythema multiforme, Stevens-Johnson syndrome and toxic epidermal necrolysis in American travelers [Miller, 1986].

When Fansidar® is administered, the two drugs act synergistically to disrupt folate synthesis. Resistance to Fansidar® has evolved worldwide, it occurs frequently and is already well established in Southeast Asia, South America and is rapidly increasing in East and Central Africa [Talisuna et al., 2004, 2002; Roper et al., 2003; TaKecki et al., 2001; Nzila et al., 2000; Plowe et al., 1998; Ronn et al., 1996]. The slow elimination of sulfadoxine-pyrimethamine (100 - 200 hours, respectively) has an advantage in providing for chemoprophylaxis after treatment but on the other hand this slow elimination favours the selection of resistant parasites [Watkins and Mosobo, 1993].

A study by Nzila and others showed that patients who are at the risk of high treatment failure are those infected with the *dhps* double mutant (Gly-437/Glu-540) and *dhfr* triple mutant (Asn-108/Ile-51/Arg-59). This quintuple mutant has been suggested as the most specific molecular marker for failure of sulfadoxine-pyrimethamine treatment of uncomplicated *P. falciparum* malaria [Kublin et al., 2002].

#### **1.8.2.4. Chlorproguanil-dapsone**

Another fixed combination of low cost, chlorproguanil-dapsone (LAPDAP) was investigated due to the increasing resistance to sulfadoxine-pyrimethamine. Dapsone, like sulfadoxine, also acts on the folate synthesis pathway by competitively inhibiting DHPS while chlorproguanil inhibits DHFR through its active metabolite chlorcycloguanil [Nzila et al., 2000]. Although resistance to LAPDAP has developed, it is less than that seen for Fansidar. This may be attributed to the shorter half-life of LAPDAP (12 - 48 hours) as compared to about 100 – 200 hours of Fansidar [AHFS Drug Information, 2000; Winstanley et al., 1997].

#### **1.8.2.5. Atovaquone-proguanil**

In 2000, Malarone® (atovaquone-proguanil) was introduced in fixed combination and has a half-life of 12 - 48 hours. Atovaquone is a selective inhibitor of parasite mitochondrial electron transport while proguanil inhibits DHFR via the active metabolite, cycloguanil [Srivastava & Vaidya, 1999]. Proguanil, independent of its metabolism to cycloguanil, has been shown to enhance the activity of atovaquone [Srivastava et al., 1999; Thapar et al., 2003].

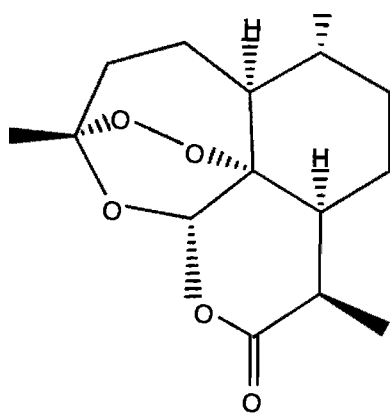
Atovaquone is an analogue of ubiquinone, an electron-carrying co-enzyme which is involved in cytochrome bc<sub>1</sub> complex of the mitochondrial electron transport chain [Srivastava & Vaidya, 1999]. Several metabolic enzymes are linked to the mitochondrial electron transport chain via ubiquinone and thus they are indirectly inhibited by atovaquone. The inhibition of these enzymes leads to the inhibition of

nucleic acid synthesis and the synthesis of biological energy, ATP (adenosine triphosphate).

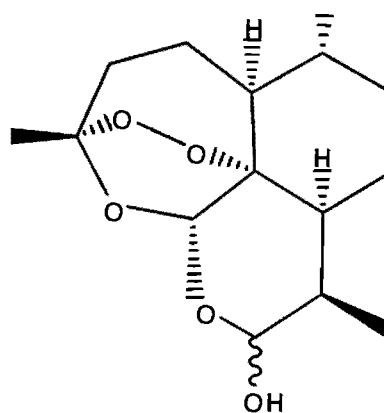
Malarone® has high efficacy against sensitive strains of *P. falciparum*, including multi-drug resistant strains, however resistance to Malarone has been reported [Farnert et al., 2003; Fivelman et al., 2002]. Atovaquone resistance has been shown to be due to single point mutations in the cytochrome b gene (*cytb*) [Korsinczky et al., 2000; Vaidya and Mather, 2000]. This may be the gene that confers resistance to Malarone® but there may also be other mechanisms since *cytb* could not be detected in one patient where there was treatment failure after Malarone® administration [Farnert et al., 2003]. Resistance to cycloguanil occurs with mutations at codon 16 (Ala→Val) and codon 108 (Ser →Thr).

### 1.8.3. Artemisinin

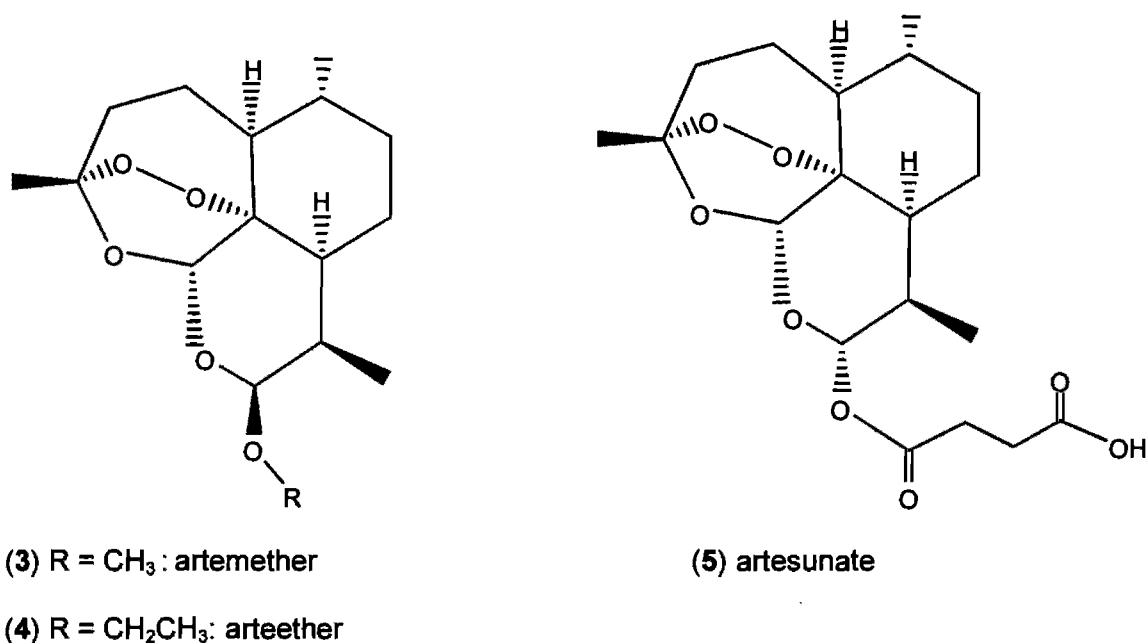
Artemisinin (qinghaosu) was isolated in 1972 from the leaves of *Artemisia annua*, a Chinese plant used traditionally as herbal tea and as a treatment for fever and malaria for over 2000 years [Li and Rieckmann, 1992]. Artemisinin and its derivatives (figure 1.9) are highly potent antimalarials which are active against multi-drug resistant malaria [Klayman, 1985; Balint, 2001].



(1) artemisinin



(2) dihydroartemisinin



**Figure 1.9.** Chemical structures of artemisinin and its derivatives (1 – 5).

Artemisinin and its derivatives are used for the treatment of severe malaria and are very effective in rapidly reducing the parasitaemia and fever. Artemisinin can reduce parasite loads within 48 hours, has a short half-life of 13 hours when taken orally [van Aghtmael et al., 1999] and cures falciparum malaria in 7 days. The artemisinin-based drugs have the ability of an excellent safety profile, short half-life and some gametocidal activity.

Artemisinin is a sesquiterpene lactone endoperoxide. It has been suggested that the antimalarial activity of artemisinin-based drugs is due to the peroxide group, in the form of a 1,2,4-trioxane, which involves the generation of an oxidative stress [Meshnick et al., 1996; O'Neill et al., 2001]. It was hypothesized that artemisinsins specifically inhibit *P. falciparum* sarcoplasmic/endoplasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA) because artemisinin is structurally similar to thapsigargin, an inhibitor of SERCA [Eckstein-Ludwig et al., 2003]. Another proposed mechanism is that, after activation, artemisinin causes local production of reactive oxygen species and depolarization of the mitochondrial membrane [Li et al., 2005].

Despite the widespread clinical use of artemisinin and its derivatives in the treatment of falciparum malaria, resistance to these drugs has not been reported. This has created the notion that resistance to artemisinin does not occur but evidence that artemisinin resistance can develop is now accumulating. *In vitro* resistance to artemether has been observed in isolates from French Guiana [Jambou et al., 2005]. An *in vivo* study has shown that stable resistance to artemisinin develops after selection pressure [Afonso et al., 2006] and a clinical study by Menard et al. has demonstrated that there is a link between reduced *in vitro* artemisinin susceptibility and loss of clinical efficacy of artesunate monotherapy. In light of this, the WHO has requested pharmaceutical companies to end the marketing and sale of 'single-drug' artemisinin malaria medicines [WHO mediacentre, 2006]. It is therefore crucial that the decreased *in vitro* susceptibility and the potential emergence of resistance to the artemisinins be thoroughly investigated.

#### **1.8.3.1. Artemisinin-based Combination Therapy (ACT)**

Although artemisinin and its derivatives are effective against multi-drug resistant parasites, their short half-lives lead to recrudescence commonly encountered after treatment with these drugs. Recrudescence rates of 44% - 54% for 3 day dosing regimens have led to recommendations that artemisinins be used in combination with drugs with long half-lives [White & Olliaro, 1996; van Aghtmael, 1999]. The introduction of ACT has shown to increase cure rates and decrease the development of drug resistance [White and Olliaro, 1996; van Aghtmael et al., 1999; White 1999]. The WHO has recommended that countries with high resistance to conventional drugs should use ACT [WHO, 2001] and most countries have now switched to artemether-lumefantrine as first-line treatment of uncomplicated malaria [Olumese, 2006].

There is concern that artemisinin derived compounds may result in neurotoxicity, based on animal studies that demonstrated that artemisinins can damage brain stem nuclei of the auditory centers [Nontprasert et al., 2002]. A number of studies

have investigated these claims [Toovey et al., 2004; Kissinger et al., 2000; van Vugt et al., 2000; Price et al., 1999] but the findings gave conflicting reports and some studies did not report on important tests, which are required to reliably assess hearing [Winstanley & Molyneux, 2004]. Novartis Pharmaceuticals Corporation said that their safety database revealed no significant adverse effects of co-artemether treatment on the auditory system therefore they found no evidence that co-artemether is associated with hearing impairment [Reinhart and Hughson, 2005].

However, there is a consensus that further studies need to be undertaken to provide evidence concerning ototoxicity and neurotoxicity of artemisinins, which are undoubtedly clinically effective. Thus, there is no convincing evidence that artemisinin derivatives results in neurotoxicity in humans. It is therefore crucial that safety issues concerning the use of artemisinin derivatives are resolved to prevent any impairment that might arise due to the use of these drugs.

## **1.9. Eradication and Control of Malaria**

Efforts to eradicate malaria began in the 1950s under the WHO and were successful, but by the 1970s the disease was resurgent again [Onori, 1993]. Malaria control and eradication is hindered by resistant strains of *P. falciparum* parasites to commonly used antimalarial drugs, as well as cross-resistance to structurally unrelated drugs, resistance of mosquitoes to insecticides, the rise of malaria transmission and the lack of an effective vaccine [WHO, 1989; Hyde, 2002; Ito et al., 2002].

Due to multi-drug resistant strains of *Plasmodium*, which are now becoming increasingly prevalent around the world, antimalarial drugs that are commonly used are losing their effectiveness [Hyde, 2002; Winstanley, et al., 2002; Wongsrichanalai et al., 2002]. Another contributing factor is the lack of industrial

interest in developing malaria vaccines for poor endemic countries especially for the asexual stage and for blocking transmission [Kilama, 2003].

It is very important to understand resistance mechanisms involved so as to develop new drugs that would minimise or circumvent drug resistance. Knowledge of resistance mechanisms may also identify new targets for drug development [Klokouzas et al., 2003].

### **1.9.1. Mass Administrations of Antimalarial drugs for Malaria Control**

In an attempt to combat resistance, combination therapy has been used. For more than 70 years mass administrations of antimalarial drugs have been used to control malaria. Mass drug administrations have proved to be ineffective in stopping the transmission of malaria, however, in reducing malaria morbidity and possibly mortality they were effective [von Seidlein and Greenwood, 2003]. This form of treatment had successes and failures. The mass drug administrations could once again be used to control malaria as shown by the development of artemisinin-based combination therapies that are less likely to induce drug resistance and which reduce infectiousness in treated patients.

The control of malaria depends on the development of new, potent and affordable antimalarials to developing countries where malaria is prevalent. The spread of malaria could be controlled by rendering the mosquitoes ineffective since they are obligatory vectors. This would help in blocking malaria transmission [Ito et al., 2002]. There is also a need for new structural classes of antimalarial agents including those from plants with novel and different mechanisms of action. New malaria control techniques, new strategies to combat malaria and new low cost antimalarial drugs that are effective against multi-drug resistant *P. falciparum* strains are also urgently needed.

Malaria transmission by mosquitoes has also been controlled by residual insecticides such as DDT (dichlorodiphenyltrichloroethane), which has residual



activity for months. Measures have also been taken to prevent human-vector contact by using insecticide treated bed-nets and topical insect repellents such as diethyltoluamide [Cook, 1996].

### **1.9.2. Search for new antimalarial drugs**

Malaria treatment is limited to schizontocidal therapy and to the relief of symptoms such as fever, headache and hypoglycaemia [Voetseder et al., 2004]. The availability of the complete genome sequence for *P. falciparum* will facilitate the development of new drugs for malaria. The genome sequence will enable understanding of host-pathogen interaction and the identification of new therapeutic targets through proteomic-genomic studies of this parasite [Florens et al., 2002; Gardner et al., 2002].

Few new antimalarials that are cost effective and potent towards drug-resistant parasites are being developed [Winstanley et al., 2002]. The number of innovative products from big pharmaceutical companies declined relative to that of smaller companies. The low productivity in pharmaceutical research could be attributed to the fact that potential productive research inquiries have been abandoned because of the focus being on the benefits to the company instead of health [Taylor, 2003].

### **1.9.3. Scope of the Project**

The emergence of resistance to currently available antimalarial drugs has made it difficult for effective treatment and eradication of malaria. There is an urgent need to address this problem. The discovery of new antimalarials through organic synthesis or from natural sources is needed.

Research has explored traditional medicine as a potential source for new and novel antimalarial drugs. Scientific follow-up of plants known for their traditional medicinal uses has led to the discovery of a high number of useful plant-derived

drugs. The antimalarial drugs, quinine and its derivatives originate from the *Cinchona* bark while artemisinin and its derivatives originate from *Artemisia annua*. More than 50% of all the drugs used clinically in the world are natural products and their derivatives [van Wyk et al., 1997; Farnsworth et al., 1985].

In searching for new antimalarials, this project investigated a traditional medicine plant, *Dicoma anomala*, which is used for the treatment of fevers and other ailments in Southern Africa. The aim of this project was to isolate antiplasmodial compounds from *Dicoma anomala* and the objectives were:

- To screen the leaf and root extracts of *D. anomala* for antiplasmodial activity against the CQS strain of *P. falciparum*.
- To isolate antiplasmodial compounds from *D. anomala* and screen them *in vitro* against the CQS and CQR strains of *P. falciparum*.
- To determine the chemical structures of the compounds using spectroscopic methods such as Mass Spectrometry and NMR.
- To determine the cytotoxicity of the compounds on CHO cells.

## ***CHAPTER 2***

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### **TRADITIONAL MEDICINE**

## **2.1. Traditional Medicine and Traditional Healing**

The well-being of people in ancient times depended on traditional medicines. Traditional medicines are mainly medicinal plants growing in the wild and used by traditional healers in different ethnic groups. Some of these plants have been used for a long time and are still used today [Mukherjee, 2002].

After years of the introduction of Western medicine, people, in particular, those in rural areas of Latin America, Asia and Africa still consult traditional healers for their ailments. This is because traditional medicine has formed part of their tradition and culture but most importantly, traditional medicines are affordable and easily available than modern medicine. It is estimated that 80 % of people in developing countries use traditional medicine [WHO, 2002] as a source of primary health care where modern health care facilities are lacking or are not easily accessible.

In addition to cultural and traditional beliefs, the use of traditional medicine in developing countries could be attributed to traditional medicine having fewer adverse effects and that no resistance to whole-plant extracts has been recorded [Willcox & Bodeker, 2000]. Complaints of side effects are less frequent in patients given herbal medicines than in the case with allopathic medicines. Common complaints are gastrointestinal disturbances with oral medicines and sneezing with inhalants [Edinburg, 1998; Mukherjee, 2002].

Due to their traditional beliefs, people residing in urban areas still consult traditional healers even though western medicines are easily available. Patients will normally seek help from medical doctors for a particular disease and when their health deteriorates or does not improve, a traditional healer is consulted [Katz & Kimani, 1982; de Wet, 1998]. Some consult the traditional healers before or after consulting the medical doctor. There are still those patients who only consult traditional healers [van Wyk et al., 1997; Kale, 1995].

Traditional healing is intertwined with religion and cultural beliefs and the healing practices vary widely between different African countries. Traditional healers believe that the western medicine treats symptoms, while their herbal remedies cure the body off the sickness. Traditional healing has a holistic approach to illness since not only the physical condition is catered for, but also the psychological, spiritual and social aspects of individuals, families and communities [Abdool Karim et al., 1994] are addressed.

Traditional healers when consulted are the first to tell the patients of what they are suffering from and the patient will admit or deny this and give details. The traditional healer will then throw bones (though not all traditional healers can do this) that will give him/her more information about the patient and the disease or the problem. Traditional medicines are always paid for but the payment is not fixed and it varies according to sickness. Some traditional healers will state that if the medicine is not paid for, it will not work. In addition to the payment of medicinal herbs, a patient may be asked to bring or slaughter an animal like a white chicken, a goat or a sheep. Slaughtering of animals is mostly done to appease the ancestors and this is served together with home-brewed beer [Katz & Kimani, 1982].

Although traditional medicine is affordable, in some instances the payment may be higher than that paid for biomedical treatment. In this case a patient can pay in small amounts or pay an initial fee and the rest of the fee is paid when treatment is complete or when the patient is healed [Green & Makhubu, 1984; Obbo, 1996].

If modern medicines have failed to cure a patient or do not improve a patient's health, this is attributed to the problem being due to a spirit of an unhappy ancestor or due to witchcraft [Gessler et al., 1995; Felhaber & Mayeng, 1997]. The illness can also be caused by the transgression of certain taboos and societal norms [Yangni-Angate, 1991]. The source or cause of illness is removed

by rituals to appease the ancestors, the neutralization of the sorcerer in cases of witchcraft or by using herbal mixtures.

It has been strongly suggested that traditional healers be integrated into the modern health since they could be of great help in the fields of psychology, psychiatry and in primary health care. Success in treating psychological problems using traditional medicines is well known and often recognized [Pearce, 1982; Kale, 1995].

Traditional healers are popular for providing medical care in a personal way. The reasons people gave for visiting traditional healers are that the consultations with doctors or hospital staff are too short, little opportunity to express their own concerns and being given medicine without explanation as to the cause of their illness [Gessler et al., 1995]. Traditional healers spend more time with their patients and take time to get involved in the patients' sickness. They have no set opening times, work throughout including public holidays and are within the communities and in most instances speak the same language and have the same culture.

Medicinal plants are not only obtained through traditional healers. Mild ailments arising within a family are treated using medicinal plants grown in the family backyard [Madureira et al., 2002]. They can also be purchased from market places and road-side stalls. Many of the people who sell these plants are not necessarily herbalists or traditional healers but are people with the knowledge of traditional medicinal plants. Information on the use of medicinal plants can be obtained in two ways. One is by literature search whereby there are no problems with intellectual property rights since the information is in the public domain. Another one is by enquiring from elderly folk, the community and traditional healers, an ethnobotanical approach. This is where intellectual property rights become an important issue. Information on medicinal plants is given to a person who the community or traditional healers can trust because the healers are reluctant to give away their secret plants or methods [Soerjarto, 1996].

The knowledge of medicinal plants is passed on to other generations in two ways. One is by word of mouth from one generation to another and the other one is by training of traditional healers [van Wyk et al., 1997].

## **2.2. Collection of Medicinal Plants**

Traditional healers collect plants under the guidance of an ancestral spirit [van Wyk et al., 1997]. These plants are collected for a particular patient or a particular disease at a certain time and location according to the information the healer received through a dream. Some plants are collected at night when they are most potent. Chemical constituents are known to vary diurnally [Edinburg, 1998]. The locality, type of soil and its moisture, time of day, the aspect of the mountain slope and the season of collection play a very important role in the chemical constituents of plants [van Wyk et al., 1997]. Chemical constituents in plants vary because of the age of the plant, the season, photo-periodicity and geographical area of collection [Farnsworth et al., 1985]. These factors, known to scientists, are also known to traditional healers in influencing the potency of their medicinal plants.

## **2.3. Plant Parts Used in the Treatment**

Different parts of the plant are used for medicinal purposes, the whole plant is rarely used. Plant parts used by traditional healers, pharmaceutical and commercial industries are the roots, stems, leaves, barks, bulbs, rhizomes, tubers, fruits, seeds, flowers, gums, exudates and nectar. Leaves and twigs are normally used together. Chemical compounds in a plant are not equally distributed and one part may contain compounds that are not found in other parts of the plant. This is what makes other parts of the plant to be toxic or non-toxic and active or inactive than the other parts. Some parts may be more toxic or more active than the others [van Wyk et al., 1997].

## **2.4. Preparation Methods for Medicinal Plants**

Plant material may be used fresh while most are used dry. The plant material is cut into small pieces and dried in the sun or in the shade away from direct sunlight. The dried plant material is then stored as it is or is ground to powder and stored in newspapers, paper bags, glass jars and tin cans. The plant material is prepared in water as decoctions or infusions. Infusion is maceration of the plant material in cold or boiling water for a short period of time. Some plant materials are prepared as inhalations (respiratory problems), lotions (skin application), ointments (wounds and skin application), enemas (rectal injections) powders and pastes (skin application and oral intake) and as snuffs [van Wyk et al., 1997; Hedberg & Staugård, 1989]. Instructions on the use of the remedy prepared by the healer are given verbally to the patient.

Commercially, the plant material is available as extracts of fractions which contain active ingredients, inhalations which contain volatile ingredients, lotions, nasal drops, ointments, tinctures containing active ingredients in alcohol and snuffs. These products are marketed as standardized products which have been tested for safety and efficacy. Medicinal plants are administered orally, sublingually, rectally, topically, nasally, by smoking, by steaming them for inhalation, by bathing in water mixed with herbal mixtures for maintaining good health or for treating skin diseases [van Wyk et al., 1997; Kale, 1995]. Some medicinal plants are placed in a small incision in the skin to enhance the onset of action [Edinburg, 1998].

## **2.5. Traditional Medicine: A Global Perspective**

Traditional medicine was outlawed in some countries during colonial times [Obbo, 1996]. Today traditional medicine and traditional practitioners are recognized and respected in most countries while in others they are merely tolerated. In China, Democratic Republic of Korea, the Republic of Korea and Vietnam traditional medicine has been integrated into the national health system



[WHO, 2002]. Countries such as Mali, Nigeria, Ghana, India, Sri Lanka, Canada and the United Kingdom among others recognize traditional medicine and have a national policy on traditional medicine [WHO, 2002]. Japan, Germany and USA do not have a national policy on traditional medicine but regulate traditional medicine and herbal products [WHO, 2002]. In Cuba traditional medicine is illegal [Tabuti et al., 2003].

The World Health Organization supports the integration of traditional medicine in the health care system. WHO established the Traditional Medicine Programme (TRM) in 1977 to help the developing countries where most people depend on traditional medicine to incorporate it into national health systems. In 1978, the WHO made a declaration at Alma-Ata, committing itself to implementing the primary health care strategy by employing useful methods such as indigenous practices and mobilising resources such as traditional practitioners and birth attendants [WHO, 1978].

WHO recognizes that traditional medicine is an acceptable, safe and economically feasible way to achieve health coverage for all. WHO believes that integration of traditional medicine and orthodox medicine into the national primary health care system would increase manpower and therefore increase health coverage [WHO, 1978, 2002].

Before being integrated or offered an equal status to the orthodox medicine, traditional healers would have to undergo basic training in hygiene and orthodox healthcare practices to serve as primary health care workers. This would require the formulations of policies, the provision of legal recognition and social equality for traditional healers. All healers would need to be registered including their premises of work and clinics. The training of traditional healers would be standardized and laws and regulations for traditional healers codes of conduct would be established [WHO, 1978, 1995, 2002].

The traditional healers need to be registered to traditional healers associations so that they can be easily identified for inclusion in the public health care system and for regulation and monitoring. The identification, registration and regulation of all healers is very important to check for harmful practices and eliminating dishonest practitioners. This will ensure a high level of professional ethics and practice and the avoidance of dangerous and damaging practices [WHO, 1978; Abdool Karim et al., 1994].

WHO support for research and development in all fields of traditional medicine is shown through its collaboration with a global network of institutional centres. It is also involved in the research of medicinal plants through its programs, Traditional Medicine Programme and Research and Training in Tropical Diseases (TDR). Research on traditional medicine is also being carried out at universities, institutes and industry.

## **2.6. Traditional Medicine and Traditional Healing in South Africa**

Medicinal plants form an important part of the South African cultural heritage and they contribute to primary health care throughout the country. South Africa has over 30 000 species of higher plants. The Cape region, with nearly 9 000 species, is rich in species and has the most diverse flora on earth. Of the 3 000 species of plants used medicinally in SA, 350 species are commonly used and traded medicinal plants. South Africa has a local and a global market for medicinal plants. Some of these plants include Cape aloes (*Aloe ferox*), buchu (*Agathosma betulina*) and devil's claw (*Harpagophytum procumbens*) [van Wyk et al., 1997].

With the development of phytomedicines, it is important and necessary for the cultivation of plants on farms for their sustainable use. Cultivation of plants ensures that rare and valuable plants are conserved from extinction. Some of these plants include pepper-bark (*Warburgia salutaris*), wild ginger

(*Siphonochilus aethiopicus*) and buchu (*Agathosma betulina*) [van Wyk et. al., 1997].

Traditional healers who are believed to be spiritually empowered are commonly known as 'dingaka' (Setswana, Sesotho and Sepedi), 'izinyanga' and 'isangoma' (Zulu), 'ixwele' and 'amaqira' (Xhosa), 'bossiedokter' (Khoi-San) [van Wyk et al., 1997]. Inyangas are herbalists and 90% of them are male, whereas isangomas are diviners who determine the cause of illness by ancestral spirits and 90% of them are female [van Wyk et al., 1997]. Most healers nowadays practice both arts of healing. Apart from healing by dingaka or isangoma there are other mediums of healing such as healing offered by intuitives, dreamers, spirit mediums, prophets and spiritual healers 'abathandazi' who are faith healers healing by prayer, holy water or ash. There are also traditional birth attendants who are elderly women who carry out most of the deliveries in rural areas. Elderly folks especially in rural areas help to treat small ailments within their families because of their knowledge of medicinal plants [Kale, 1995; van Wyk et. al., 1997].

Traditional healers are respected in their communities and their existence in South Africa dates back to 17<sup>th</sup> century [Kale, 1995]. Compared to 25 000 medical doctors of modern medicine, South Africa has about 200 000 traditional healers who render their services to about 80% of the black population [Kale, 1995]. They are consulted for various diseases including sexually transmitted diseases, divulgence of secrets, immunization against witchcraft, prophecies of future events.

It has been reported that in South Africa, 80% of patients consult a traditional healer before consulting a medical doctor and that about 60% of all the babies are delivered by traditional birth attendants [Abdool Karim et al., 1994]. Traditional healers were banned in South Africa by the Health Act of 1974. Despite this law many organizations registered traditional healers. These organizations include the Congress of Traditional Healers of South Africa,

Southern African Traditional Healers Council, African Dingaka Association, the association of Traditional Healers Council and the African Skilled Herbalists Association [Freeman & Motsei, 1992].

The South African government recognizes the use of African traditional medicines and the research on traditional medicine at universities and science councils. In 2003 the Traditional Health Practitioners Act was implemented to make provision for control of the registration, training and practices of traditional health practitioners in the Republic of South Africa and to register African traditional medicine and regulate them under the Medicine Control Council [Department of Health]. The Department of Health has also established the National Reference Centre to evaluate therapeutic claims of traditional medicine.

## **2.7. Medicinal Plants as Source of Drugs**

It is estimated that there are 250 000 plant species of higher plants in the world, of which 220 000 are flowering plants [Wilson, 1988]. Approximately 119 chemical agents, which are clinically used worldwide were obtained from flowering plants and less than 90 species of the medicinal plants have been identified as the source of these drugs [Farnsworth et al., 1985]. The World Health Organization estimates that about 20 000 plant species are used medicinally throughout the world in about 90 countries, of which 250 species are widely used or have been analyzed for the identification of active components [WHO, 1978]. This means that most species have not been studied chemically and pharmacologically. The greatest biodiversity in the world is found in the tropical rain forests [Wilson, 1988].

Natural products are products derived from plant, animal, microbe and mineral sources [Soejarto, 1996; Edinburg, 1998]. Microorganisms and fungi have provided antibiotics such as penicillin, neomycin, streptomycin and cephalosporin-C. Products obtained from animals include the human growth hormone, ovine and porcine insulin [Mukherjee, 2002]. There are therefore good

chances of finding new drugs through the investigation of natural products. In a survey done between 1959 and 1980 in the US it was estimated that about 25% of all medicines dispensed from community pharmacies contained plant extracts or their active principles [Beecher et al., 1989]. Previous research results showed that about 40% of all drugs approved between 1983 and 1994 were natural products or derived from natural products [Cragg et al., 1997].

The structural diversity in natural products increases the chances of finding a compound with activity [Harvey, 1999]. Some compounds are not confined to one species or one family. Compounds related or similar to the ones isolated from a particular plant could also be isolated from varieties within the same species, species of the same genera and genera within the same family. This is advantageous when that particular compound has activity since the bioassays and screening in higher animals would not be limited by the availability of the plant material.

The bioactive molecules found in medicinal plants are secondary metabolites which are synthesised by the plant for its survival against predation and infection [Williams et al., 1989]. These biologically active secondary metabolites often have highly complex chemical structures. Active ingredients that have been isolated from medicinal plants include glycosides, amino acids, lectins, tannins, quinines, flavonoids, terpenoids, alkaloids and coumarins.

Drugs that have been derived from plants include quinine (*Cinchona spp*) an antimalarial drug, vinblastine and vincristine (*Catharanthus roseus*) anticancer drugs, aspirin (*Salix spp*) an analgesic, atropine (*Atropa belladonna*) an anticholinergic and morphine (*Papaver somniferum*) an analgesic that is mostly converted to codeine which is used to treat headaches and is an ingredient of cough syrups. Aloe bitters from *Aloe vera* are used as laxatives and aloe gel which has healing properties is obtained from (*Aloe ferox*) [Farnsworth, 1988; van Wyk et al., 1997].

Not all medicinal plants or their products are used as extracts or pure drugs, some are used as starting material for the semi-synthetic preparation of other drugs. The synthesis of oral contraceptives and other steroid hormones are such examples. Plant steroids, diosgenin from *Dioscorea spp* and hecogenin from the leaves of *Agave sisalana* are used in the synthesis [Chevallier, 1996].

## 2.8. Medicinal Plants as Source of Antimalarial Drugs

There is a rapid spread of *Plasmodium falciparum* resistance to available antimalarials, and this has called for a need for alternative antimalarials. Medicinal plants are used in the treatment of malaria in Africa, Americas and Asia [Phillipson et al., 1987]. However, few scientific data are available to assess the efficacy of these medicinal plants. Table 2.1 shows traditional medicinal plants that are used to treat malaria and fevers in South Africa. In search for new antimalarial drugs, these medicinal plants have to be thoroughly investigated and their efficacy confirmed. Some of the medicinal plants that are used traditionally for the treatment of fevers and malaria have shown antiplasmodial activity *in vitro* against *P. falciparum* (table 2.2).

It is therefore, important that medicinal plants that are known to have antimalarial properties are investigated in order to determine their potential as sources of new antimalarial drugs [Gessler et al., 1994]. There is also a need to confirm the therapeutic effects of traditional medicine through controlled studies [Mukherjee, 2002]. The search of drugs from medicinal plants and the recognition and validation of traditional medical treatments could therefore lead to new and better interactions for controlling malaria.

Since plant species provide with chemical diversity, traditional medicine is likely to lead to compounds or classes of compounds that can be developed as new antiplasmodials [Wright and Phillipson, 1990]. Antiplasmodial activity has been observed in different classes of plant metabolites as shown in table 2.3.

Indigenous plants therefore play an important role in being a source of novel antiparasmodial compounds.

**Table 2.1.** Plants used traditionally in South Africa to treat malaria and fevers.

<b>Botanical Name</b>	<b>Family</b>	<b>Local Name</b>	<b>Medicinal Uses</b>
<i>Acacia burkei</i> Benth.	<b>Fabaceae</b>	Mkhaya	The roots are burnt and the ash is mixed with an equal amount of <i>Bopusia scabara</i> herbs and a little fat or oil for eruptive fevers. The plant is used for dysentery, conjunctivitis and diarrhoea [Bryant, 1966; van Wyk et al., 1997].
<i>Dicoma anomala</i> Sond	<b>Asteraceae</b>	Tlhonya, Hloeya, Nyongwana	Colic, toothache, diarrhoea, colds, stomach ailments [van Wyk et al., 1997; Hutchings, 1996]. The leaf and twig extracts are used for the treatment of malaria and fever [Neuwinger, 2000].
<i>Harpagophytum procumbens</i>	<b>Pedaliaceae</b>	Sengaparile	Emetic, blood purifier, eczema, acne and for sprains and fractures [van Wyk et al., 1997].
<i>Leonotis leonurus</i> (L.) R.Br.	<b>Lamiaceae</b>	Klipdagga	Used for the treatment of skin diseases, coughs and colds, influenza, bronchitis, asthma, high blood pressure, epilepsy, headaches and viral infections [van Wyk et al., 1997].
<i>Lippia asperfolia</i> Reichb.	<b>Verbenaceae</b>	Umsuzwane	Febrile complaints, measles, gangrenous rectitis and as an antidote to food poisoning [Bryant, 1966, van Wyk et al., 1997].
<i>Mentha longifolia</i> (L.) L subsp. <i>Capensis</i> (Thunb.) Briq	<b>Lamiaceae</b>	Koena	Cold, coughs, fevers, asthma, indigestion, headaches, hysteria, respiratory ailments, flatulence, delayed pregnancy, painful menstruation, urinary tract infections [van Wyk et al., 1997].

**Table 2.2.** Examples of traditional medicinal plants that have shown *in vitro* antiparasmodial activity against *P. falciparum* strains.

<b>Botanical Name</b>	<b>Medicinal Uses</b>	<b>Extract screened</b>	<b>In vitro Antiparasmodial activity</b>	<b>Reference</b>
<b><i>Lippia javanica</i></b>	Malaria, headaches, diarrhoea, coughs and colds in South Africa	Acetone extract of the leaves	IC <sub>50</sub> = 4.26 µg/ml against CQ-resistant strain PfUP1	Prozesky et al., 2001
<b><i>Casearia elliptica</i></b>	Malaria and fever in India	Ethanol extract of the leaves	IC <sub>50</sub> = 9 µg/ml against CQ-sensitive strain 3D7	Simonsen et al., 2001
<b><i>Cinchona succirubra</i></b>	Malaria and fevers in the islands of Gulf of Guinea	Ethanol extract of the bark and fractions	IC <sub>50</sub> ≤ 10 µg/ml for the crude and fractions against CQ-resistant strain Dd2	do Céu de Madureira et al., 2002
<b><i>Terminalia macroptera</i></b>	Malaria, fever, nausea and headache in Burkina Faso	Water extract of the root bark	IC <sub>50</sub> = 1 µg/ml against CQ-resistant strain W2	Sanon et al., 2003
<b><i>Coscinium fenestratum</i></b>	Malaria in Vietnam	Methanol extract of the stem	EC <sub>50</sub> = 0.5 µg/ml against CQ-sensitive strain FCR-3	Tran et al., 2003
<b><i>Croton lobatus</i></b>	Malaria in Benin	Methanol extract of the aerial parts	IC <sub>50</sub> = 0.38 µg/ml against CQ-sensitive strain 3D7	Weniger et al., 2004
<b><i>Fagara macrophylla</i></b>	Malaria and fever in central west Ivory Coast	Ethanol extract of the stem bark	IC <sub>50</sub> = 2.3 µg/ml against CQ-resistant strain FcB1	Zirihi et al., 2005



**Table 2.3.** Some of the isolated phytochemical constituents that has shown *in vitro* antiplasmodial activity against *P. falciparum* strains and *in vivo* activity against *P. berghei*.

<b>Botanical Name</b>	<b>Active constituents</b>	<b>Comments</b>	<b>References</b>
<b><i>Funtumia elastica</i></b>	Alkaloids	Significant inhibition <i>in vitro</i> .	Zirihi et al., 2005
<b><i>Stephania erecta</i></b>	An alkaloid	Moderate activity <i>in vivo</i> .	Tamez et al., 2005
<b><i>Cichorium intybus</i></b>	Sesquiterpene lactones	Complete Inhibition of parasite growth <i>in vitro</i> .	Bischoff et al., 2004
<b><i>Eurycoma longifolia</i></b>	Quassinoids and an alkaloid	Highly active <i>in vitro</i> .	Chan et al., 2004
<b><i>Azorella compacta</i></b>	Diterpenes	Active <i>in vivo</i> with 60% and 42% parasitaemia inhibition, respectively.	Loyola et al., 2004
<b><i>Milletia usaramensis</i></b>	Flavonoids	Potent to moderate activity <i>in vitro</i> .	Yenesew et al., 2003
<b><i>Gardenia saxatilis</i></b>	Triterpenes	Very good activity <i>in vitro</i> .	Suksamrarn et al., 2003
<b><i>Pleiocarpa mutica</i></b>	Alkaloids	Highly active <i>in vitro</i> . One alkaloid showed moderate activity <i>in vivo</i> .	Addae-Kyereme et al., 2001
<b><i>Hannoa chlorantha</i></b>	Quassinoids	Highly active <i>in vitro</i> . Parasitaemia suppressed <i>in vivo</i> from 4 – 7 days	François et al., 1998

## 2.9. Safety of Medicinal Plants

For herbal medicines to be considered safe they should be free from botanical contaminants (other plants or compounds from other plants), residual pesticides, fumigation agents, pathogenic microorganism and microbial toxins. They also have to be standardized.

Toxicity is of a great concern since no scientific data are available on the toxicity of herbal remedies and medicinal plants. The improper identification of herbs and medicinal plants may lead to toxicity. This is caused by individuals collecting the plant by themselves or improper botanical identification, where a wrong plant that looks similar to the correct one is collected [Mukherjee, 2002 ].

Research done in rural areas of Africa has shown that a quarter of the corneal ulcers and childhood blindness is caused by the instillation of traditional medicines used for the eye treatment [Yorston & Foster, 1994; Lewallen & Courtright, 1995].

An edible plant, *Matteuccia struthiopteris* is generally considered non-toxic, but when cooked lightly, blanched or lightly sautéed it causes gastrointestinal toxicity [Mukherjee, 2002]. Therefore, thorough cooking is required to render the plant non-toxic. Another example is *Xanthium strumarium*, a chinese herb used for the treatment of allergic rhinitis, atopic dermatitis and headache caused by wind-dampness. *Xanthium strumarium* is non-toxic when stir-fried without the spikes [Mukherjee, 2002; Dharmananda, 2002].

Another safety concern is that of people who use herbal medicines together with other drugs. These people are at risk as a result of herbal-drug interactions. For an example, the roots of *Salvia miltorrhiza* used in Chinese traditional medicine for the treatment of coronary diseases, can enhance the anticoagulant activity of warfarin, when both are taken together [Lo et al., 1994].

A great safety concern with medicinal plants is that most of the herbal products sold at herbal markets are not monitored. Arsenic and mercury have been found as constituents in Indian herbal remedies [Muzi et al., 2001; Kew et al., 1993]. Heavy metals have also been found in Chinese herbal remedies together with undeclared pharmaceuticals, which include methyltestosterone, phenacetin, chlorpheniramine and ephedrine [Ko, 1998]. Some of these metals and pharmaceuticals are added deliberately to enhance the activity of the products while some are purely as a result of contaminants.

## 2.10. Challenges of using Traditional Medicine

National policies on traditional medicine are needed for the legislation and regulation of herbal products and therapies. Once regulated, it would be easier to evaluate herbal products for safety, efficacy and quality. The identification of effective herbal therapies requires sustainable cultivation of medicinal plants if the isolated constituents cannot be synthesized. Traditional practitioners would have to be trained in order to provide reliable information for consumers on the proper use of herbal products and therapies [WHO, 2002].

The greatest problem with the use of traditional medicine is the isolation of active compounds from medicinal plants because the isolation of active compounds for *in vitro* and *in vivo* bio-assays depends on the availability of a large quantity of the plant material. This quantity is critical if large amounts of the active compounds cannot be obtained through chemical synthesis. Another challenge is that species could be lost due to the destruction of habitat. The global conservation organization, the World Wide Fund for Nature estimates that of all the total plant species, 50 000 species are being lost every year through destruction of tropical forests. Most of these species have not been tested for biological activity.

Due to deforestation, plant and animal species are heading for extinction and since the highest biological diversity is found in the tropical rain forests, species

that were going to provide with useful drugs would be lost. The continual growth of human population makes it difficult to conserve forests or natural resources due to housing, agricultural and industrial developments needed to sustain life. Sometimes this may be the end to finding lead compounds especially if the plant is rare [Soejarto, 1996].

Another contributing factor is plant gathering for herbal medicine and other natural health care products, which after a while results in over-harvesting. The knowledge of traditional uses of plants could also be lost with the gradual loss of indigenous cultures [Mukherjee, 2002]. Financial constraints on the other hand make it impossible for all plant species to be screened.

Medicinal plants are abundant, culturally acceptable and affordable in developing countries, which are rich with information on these plants. It is therefore crucial to obtain scientific data on medicinal plants that have shown to exhibit pharmacological activity.

## **2.11. Drug Discovery and Drug Development**

### **2.11.1. Drug discovery**

There are three ways which could be used to select medicinal plants for the discovery of drugs, (1) Ethnopharmacological – where plants are selected based on their medicinal use by an ethnic group, (2) Chemotaxonomical – where plants are chosen according to a chemical class in a genus or family and (3) randomised – where any plant is studied for therapeutic use [Farnsworth, 1988]. Of these, the ethnopharmacological approach offers an opportunity of discovering compounds with new structures and new activities [Malone, 1983]. According to Farnsworth et al., 1985 this is a good approach as compared to massive screening of plants for biological activity, new compounds or structures. There is another method used by pharmaceutical companies to find lead compounds from medicinal plants. This is called the 'shotgun' method whereby

thousands of compounds being from plants or from chemical synthesis are screened for biological activities or for lead compounds. While there may be a chance of finding a lead compound with this method these chances are very low and the approach is considered very expensive [Soerjarto, 1996]. Although this method is expensive, it led to the discovery of taxol from *Taxus brevifolia* by the United States National Cancer Institute. The screening programme took place between 1960 and 1982. Although taxol was the only drug discovered from all the plant extracts screened, the National Cancer Institute program played a role in the development of two antitumour drugs, vinblastine and vincristine. [Cragg et al., 1994].

During an ethnobotanical survey, medicinal plants are collected by personal contact with local traditional healers and old people who have knowledge of medicinal plants. The collection of medicinal plants is dependent on the permission by healers, village chiefs, landowners and government and it take place under the guidance of the traditional healers [Madureira et al., 2002].

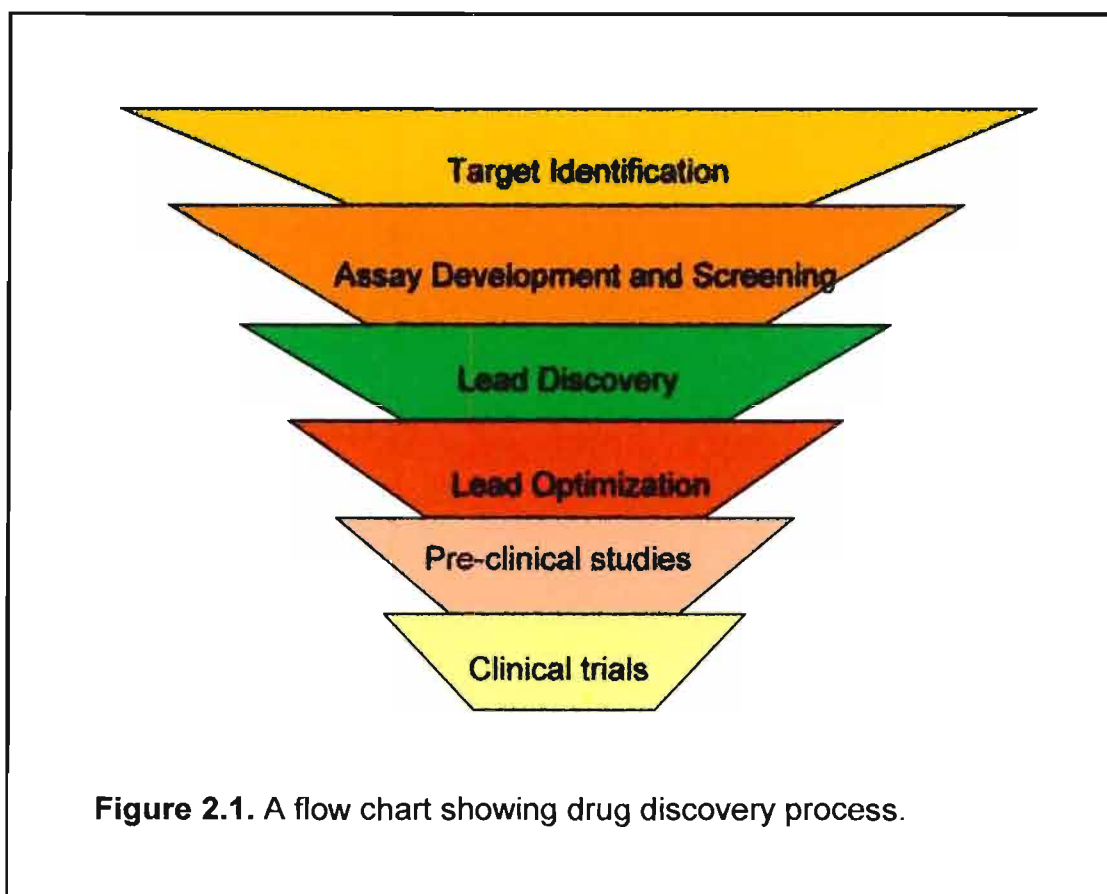
Informal interviews are conducted, recorded on tape and/or filmed. Information collected on medicinal plants includes vernacular name of the plant, plant parts used, mode of preparation, form of administration, the diseases and symptoms treated by the particular medicinal plant. Information on these plants is stored in the form of voucher number, collecting locality and date of collection [Madureira et al., 2002]. Once activity is found, medicinal plants are collected from the same location since activity will differ due to factors such as type of soil, humidity, rainfall and the amount of sunlight [Soejarto, 1996].

### **2.11.2. Drug Development**

The active compounds in the active extract are identified by purifying the extract using chromatographic methods such as Solid Phase Extraction (SPE), High Performance Liquid Chromatography (HPLC) and preparative Thin Layer Chromatography (TLC). All the isolated compounds are screened again for

activity and the promising compounds are identified by spectroscopic methods such as Nuclear Magnetic Resonance and Mass Spectroscopy.

The pure compounds are then screened for *in vivo* activity and toxicity in animal models. The compound with activity and less toxicity or non-toxic effects on the animal model is taken for clinical trials (Phase I, Phase II and Phase III) and then registered and marketed as a drug.



## ***CHAPTER 3***

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### **THE ETHNOBOTANY AND ETHNOPHARMACOLOGY OF *Dicoma anomala***



### **3.1. *Dicoma anomala* (Sond.) subspecies *anomala***

#### **3.1.1. Description of the plant**

*Dicoma anomala* is a perennial herb, which grows up to a length of 0.3 m at an altitude of 152 – 2200 m. The herb grows on hillsides and on flat grasslands [Netnou and Herman, 2003].

The *Dicoma* species is widely distributed throughout Africa. It is found in Botswana, Zimbabwe, Namibia to Democratic Republic of Congo and Uganda in the North. In South Africa it is distributed from the provinces of the Eastern Cape, Kwazulu-Natal, Mpumalanga, Limpopo, Gauteng, North West and Free State through Lesotho and Swaziland [Netnou and Herman, 2003].

*Dicoma* species belongs to the Mutisieae tribe of the Asteraceae family, one of the largest angiosperm family. The Asteraceae is only absent from the Antarctica and has about 1 535 genera and 25 000 species, which are distributed over three subfamilies and 17 tribes in the Asteraceae family [Bremer, 1994; Herman et al., 2000]. In South Africa there are about about 246 genera and 2305 species of the Asteraceae family [Herman et al., 2000]. Secondary metabolites synthesized in this family are polyacetylenes, flavonoids and terpenoids. Terpenoids in particular sesquiterpene lactones are the most studied secondary metabolite and are used as taxonomic markers [Seaman, 1982].

### **3.2. The collection of the plant**

The roots and leaves of *D. anomala* were collected from three provinces in South Africa (Eastern Cape, Free State and Limpopo) and in Lesotho during the month of October 2003. The map of the nine provinces of South Africa and the country of Lesotho are shown in Figure 3.1.

The plant was collected with the assistance of Mrs Lentsoe in Sterkspruit, a community member with the knowledge of the plant. In Lesotho we met with a



traditional healer Mr Tumelo and a community member, Mr Lekhelebane who helped us with the collection of the plant roots.



The roots of *D. anomala* from Lesotho were collected in the fields of Morifi village, in the region of Mofale's hoek. This was the second batch of plant roots collected. The first batch was collected in December 1996. The plant material was taxonomically identified and authenticated by the Department of Botany, University of Cape Town. Voucher specimens of the plant (98142) have been deposited in the herbarium of the same department. To avoid obtaining different experimental results, the second batch (batch II) of the plant material was collected in October 2003, closer to the time the first batch was collected.

In Reitz, we collected the plant roots and leaves from a traditional healer, Ms Mahlaba who had collected the plant material from a nearby farm. In Riverside, we collected the plant roots and leaves with the assistance of traditional healer Mr Seth Seroke.

### 3.3. Ethnopharmacology of *Dicoma anomala* (Sond.)

*Dicoma anomala* is known by other names in different languages (table 3.1).

**Table 3.1.** Vernacular names of *D. anomala*.

Language	Common name
<b>Setswana</b>	Tlhonya
<b>Sesotho</b>	Hloenya
<b>IsiZulu</b>	iCala alithethwa
<b>IsiXhosa</b>	Inyongwane
<b>Shona</b>	Chifumuro
<b>Manyika</b>	Chiparurangomo
<b>Afrikaans</b>	Koorsbossie, Maagbosie, Wurmbos
<b>English</b>	Toy Protea

In Lesotho, the Basotho people use root decoctions for intestinal worms, colic, diarrhoea, dysentery, toothache and as purgatives. The root decoctions are also used for gall-sickness in stock animals. The plant can also be used in powder form for sores and wounds in horses [Hutchings et al., 1996].

In South Africa, the Zulus use *D. anomala* for coughs, respiratory complaints and dysentery. They also use root decoction for children suffering from blood disorders. For sores on the head, the roots are charred and a paste applied to the sores [Hutchings et al., 1996; van Wyk & Gericke, 2000]. The Xhosas chew

the roots and swallow the sap to induce vomiting to get rid of ingested poisoned food. Whites in southern Africa use root decoction in gin for haemorrhoids or mixed with “melkbos” plants for fevers [Hutchings et al., 1996].

In Zimbabwe and South Africa the roots are used as a panacea and the plant is known as a fever remedy in other parts of southern Africa [van Wyk and Gericke, 2000]. In Tanzania, the plant is used for dysentery and as anthelmintic and in East Africa the plant roots are used for stomach complaints. In Zimbabwe, fever, abdominal pain, dizziness and cataracts are treated with plant tubers. For diarrhoea, the root powder is taken in food or a root decoction is drunk [Hutchings et al., 1996, Neuwinger, 2000]. The dose regimen is half a cup of boiled ground roots two times a day [Hedberg & Staugård, 1989].

The plant is also used to treat gonorrhoea and other venereal diseases. Powdered roots are taken with porridge to treat pneumonia. For the treatment of uterine pain, powdered tubers are inserted into the vagina and for throat problems, tuber powder with salt is eaten with porridge. Tuber infusion is used to wash the body to calm body pains and is mixed with soot and drunk for extended labour pains [van Wyk & Gericke, 2000; Neuwinger, 2000].

Abscess and dermatosis are treated by eating or sniffing leaf ash. To treat scabies leaves are eaten, and for insanity and possession a leaf decoction is drunk. An extract of leaves and twigs is drunk for the treatment of malaria, fever, epilepsy and constipation. Leaves are also used for migraines and snakebites [Neuwinger, 2000].

Very little has been reported on the pharmacology of *D. anomala*. Although it is widely used, there have been reports that the extracts are hepatotoxic [Hedberg & Staugård, 1989]. This information contradicts the claims of traditional healers that *Dicoma anomala* is a very safe traditional medicine.

### **3.4. Phytochemical information on *Dicoma anomala***

Compounds isolated from the aerial parts of *D. anomala* include diterpenes, triterpenes, sesquiterpene lactones, germacranolides, phytol, lupeol and lupeol acetate. The germacranolides and sesquiterpene lactones were also found in the subspecies of this medicinal plant [Bohlmann & le Van, 1978; Bohlmann et al., 1982; Zdero & Bohlmann, 1990]. The structures of these compounds were elucidated by spectroscopic methods including the highfield  $^1\text{H}$ -NMR spectroscopy.

## ***CHAPTER 4***

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### **MATERIALS AND METHODS**

## **4.1. Plant Collection and Extraction**

### **4.1.1. Plant collection, identification and documentation**

The roots of *Dicoma anomala* from Lesotho were collected at Morifi village, in the district of Mophale's hoek. The first batch (batch I) was collected in December 1996, the second batch (batch II) in October 2003. The plant material was taxonomically identified and authenticated by the Department of Botany, University of Cape Town. A voucher specimen of the plant (98142) has been deposited in the herbarium of the same department.

The roots and leaves of *Dicoma anomala* from Reitz in the Free State Province, Riverside in Limpopo Province and Sterkspruit in the Eastern Cape Province were collected in October 2003. The collection of *Dicoma anomala* in Reitz was done with the assistance of traditional healer Ms Maria Mahlaba, in Riverside (Limpopo) with traditional healer Mr Seth Seroke and in Sterkspruit with a community member Ms Lentsoe.

### **4.1.2. Preparation of cold extractions of the plant material**

The leaves and twigs and the roots of *Dicoma anomala* were first washed, air-dried at room temperature and ground to fine powder with a laboratory blender (Waring Commercial, Conneticut, USA).

Cold extractions of a mixture of the dried powdered leaves and twigs and the roots from the four locations were done daily by leaving the plant material overnight in a solvent, at room temperature on a mechanical shaker (Labcon, California, USA). The leaves and twigs were extracted in AR grade dichloromethane (Merck, Darmstadt, Germany) while the roots were extracted respectively, in dichloromethane and purified deionised Millipore water (Waters, Milford, USA). The plant material was extracted to exhaustion (until the solvent remained clear). The extracts were then filtered through a 320 mm Rundfilter filter paper (Schleicher & Schuell, Dassel, Germany) and the dichloromethane (DCM) extracts were concentrated under vacuum (600 mbar) at 45 °C using a rotary evaporator. The rotary evaporator consisted of a

pump Vac® V-500, a heating bath B-490, a vacuum controller V-805 and a rotavapor R-205 (BUCHI Labortechnik AG, Flawil, Switzerland). Crude extracts of the roots collected from Lesotho were also prepared with chloroform, hexane, ethylacetate, petroleum ether and methanol. The organic extracts were left to dry in pre-weighed beakers in the fume hood. The water (H<sub>2</sub>O) extracts were concentrated by freeze-drying on a -85°C Dura-Dry™ freeze-dryer (FTS Systems, Inc., New York, USA) and all the dried extracts were stored at 4°C until required.

#### ***4.1.3. Preparation of hot extractions of the plant material***

Hot extractions of the dried powdered roots from the four locations were prepared for 6 hours with DCM and Millipore water, respectively, on the Soxhlet extractor. A 1 L round-bottomed flask contained the solvent and anti-bumping granules (BDH Chemicals Ltd, Poole, Dorset, England) to prevent overboiling of the solvent. The flask was placed in an isomantle heating apparatus (Isopad Ltd, GB-Washington England) connected to a temperature controller. The isomantle heating apparatus was labelled from 1 – 5, with 5 being the high temperature. All the hot extractions were carried out with the temperature controller on number 3, which was medium heat.

Another hot water extraction of the roots was prepared on a stirrer-hotplate (ChemLab, Essex, England). The powdered roots were extracted in water, on low heat and stirred with a magnetic stirrer for 2 and 6 hours. Tables 4.1 and 4.2 show the mass of the plant material extracted and the volume of the solvent used for the extraction.

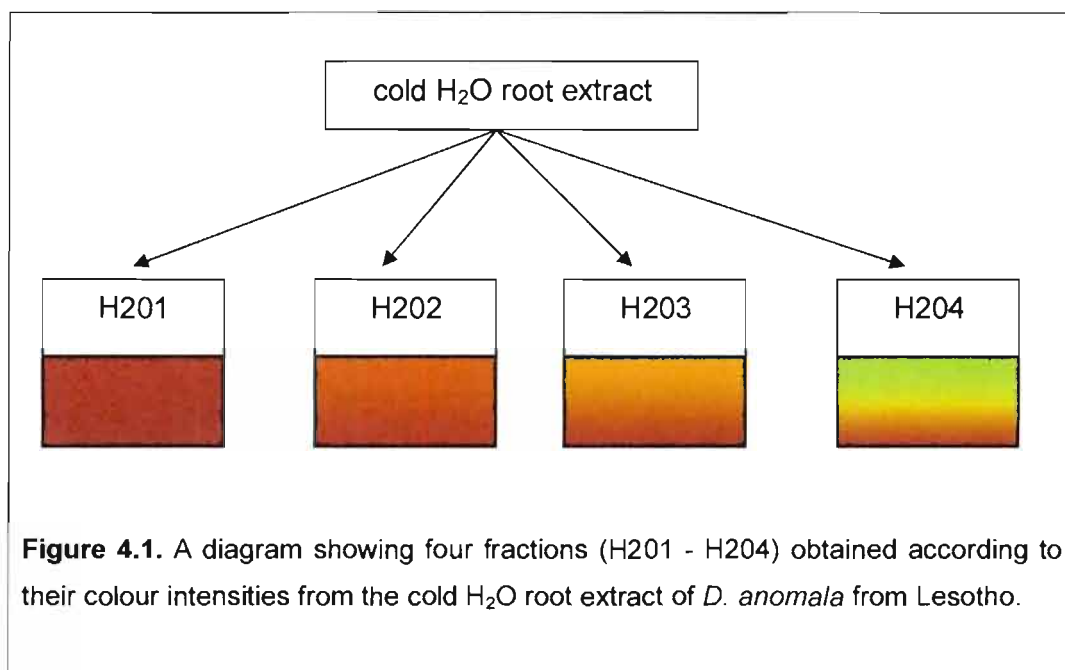
**Table 4.1.** Cold and hot extractions of the leaves and twigs and the roots of *Dicoma anomala* collected from four locations (Lesotho, Sterkspruit, Limpopo and Reitz).

<b>Cold extractions</b>	<b>Mass extracted (g)</b>	<b>Solvent</b>	<b>Volume of solvent (ml)</b>
<b>Leaves and twigs</b>			
Sterkspruit	10.69	DCM	150
Limpopo	12.37	DCM	150
Reitz	2.21	DCM	75
<b>Roots</b>			
Sterkspruit	100	DCM	500
	10.25	H <sub>2</sub> O	200
Limpopo	50.02	DCM	250
	10.40	H <sub>2</sub> O	200
Reitz	50.04	DCM	250
	10.20	H <sub>2</sub> O	200
Lesotho	200.43	DCM	1000
	5.02	CHCl <sub>3</sub>	100
	5.02	EtAC	100
	5.01	Hexane	100
	5.15	MeOH	100
	5.18	PE	100
	5.15	H <sub>2</sub> O	100
<b>Hot extractions (roots)</b>			
<b>Soxhlet: 6 hrs</b>			
Lesotho	40.0	DCM	700
	40.0	H <sub>2</sub> O	700
Sterkspruit	40.61	DCM	700
	40.50	H <sub>2</sub> O	700
Limpopo	40.22	DCM	700
	41.19	H <sub>2</sub> O	700
Reitz	20.20	DCM	350
	17.47	H <sub>2</sub> O	350
<b>Hotplate: 6 hrs</b>			
Lesotho	5.0	H <sub>2</sub> O	500
Sterkspruit	5.29	H <sub>2</sub> O	500
Limpopo	5.37	H <sub>2</sub> O	500
Reitz	5.60	H <sub>2</sub> O	500
<b>Hotplate: 2 hrs</b>			
Lesotho	5.0	H <sub>2</sub> O	500
Sterkspruit	5.24	H <sub>2</sub> O	500
Limpopo	5.50	H <sub>2</sub> O	500



#### 4.1.4. Separation of the cold H<sub>2</sub>O root extract of *D. anomala* from Lesotho

It was observed that as the roots of *D. anomala* from Lesotho were being successively extracted in water, the amount of plant material progressively decreased. This led to the preparation of a water root extract (5.16 g of the powdered roots in 100 ml Millipore water), which was carried out daily and four fractions (H201 - H204) of the water root extract were obtained as shown in figure 4.1 below. This extraction was done to determine whether the four fractions from the cold H<sub>2</sub>O root extract would display similar *in vitro* antiplasmodial activity and also to determine whether the activity would be similar or different to the cold H<sub>2</sub>O root extract that was not separated into fractions.

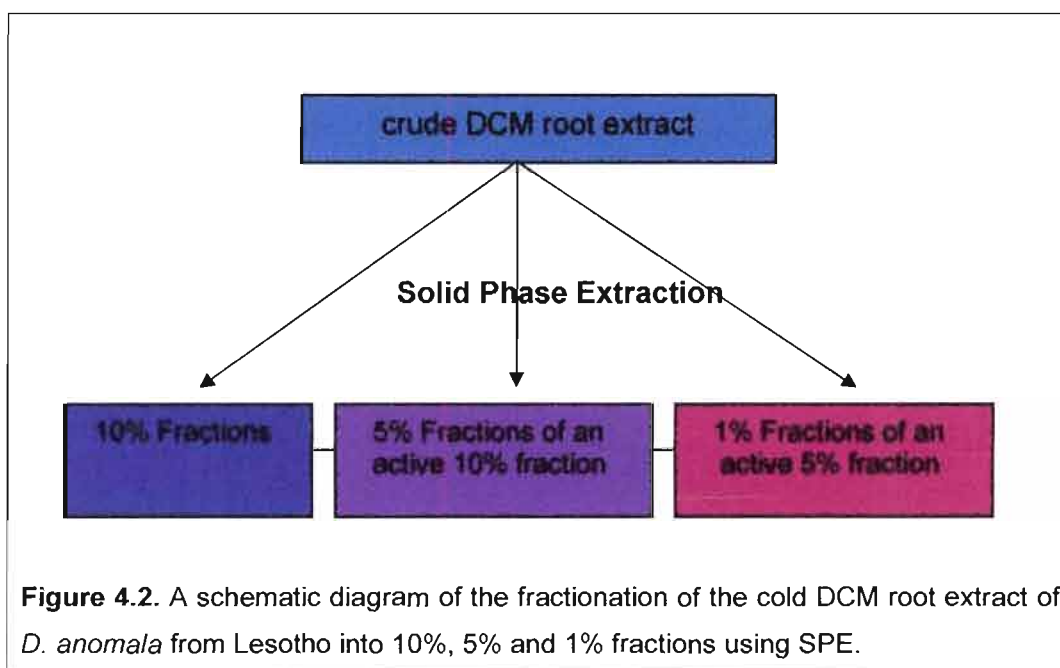


The *in vitro* antiplasmodial activity of the four water extract fractions showed that the third and fourth fractions (H203 and H204) were active. The consistency of these results was determined by repeating the extraction procedure by extracting 5.15 g of the powdered roots daily with 100 ml Millipore water. Four fractions were again obtained (H1 - H4) and were screened for *in vitro* antiplasmodial activity.

## 4.2. Solid Phase Extraction

Solid Phase Extraction (SPE) is an extraction method that uses a solid phase and a liquid phase to isolate one or more analytes from a solution. A solution is loaded onto a column and the analytes allowed to bind under vacuum. Any unbound analytes are then washed off with water and the bound analytes are then eluted with one or more solvents.

The crude DCM root extract solution was loaded onto a C18 SPE column, any unbound material was washed off with water and the bound material eluted with a step gradient of HPLC grade acetonitrile (Merck, Darmstadt, Germany) and Millipore water (MeCN:H<sub>2</sub>O). All samples were collected and concentrated by rotary evaporation and freeze-drying. Figure 4.2 below shows the SPE of the crude DCM root extract.



**Figure 4.2.** A schematic diagram of the fractionation of the cold DCM root extract of *D. anomala* from Lesotho into 10%, 5% and 1% fractions using SPE.

### 4.2.1. Preparation of crude DCM root extract from Lesotho for SPE

The crude DCM root extract (cold extraction) from Lesotho was separated by SPE, eluting with a step gradient of decreasing polarity of a mixture of water and acetonitrile (MeCN:H<sub>2</sub>O) mobile phases. An initial stock solution of 13

mg/ml in MeCN was sonicated for 1 hour in a UMC5 sonicator (Ultrasonic Manufacturing Company (Pty) Ltd, Krugersdorp, South Africa). The stock solution was diluted with water to 4 mg/ml solution, which was sonicated for another hour to make sure that the crude DCM root extract was dissolved. The solution was filtered through a 320 mm Rundfilter filter paper to remove any particulate matter.

#### **4.2.2. SPE fractionation of the crude DCM root extract from Lesotho**

The fractionation of the crude DCM root extract of *D. anomala* from batch I and batch II of the plant root material was carried out using the Isolute C18 (EC) cartridge of mass absorbent 10 g and 70 ml capacity (International Sorbent Technology, Glamorgan, United Kingdom). To facilitate binding of compounds, the column was conditioned sequentially with 50 ml AR grade methanol (Merck, Darmstadt, Germany) and 50 ml Millipore water. A 50 ml aliquot of the 4 mg/ml crude DCM root extract solution was then applied onto the column and eluted under vacuum at a flow rate of 17 ml/min. The eluant (percolate) was collected and the unretained material was rinsed off from the column with 200 ml water (water wash). The retained material was separated using 200 ml volumes of a step gradient of 10% increments of MeCN:H<sub>2</sub>O in the range 0% - 100%. The final 100% MeCN mobile phase was followed by a 100 ml of AR grade acetone (acetone wash) (Merck, Darmstadt, Germany) to rinse off any remaining material from the column.

The water wash fraction was concentrated by freeze-drying and the percolate and fractions 10 - 90 were concentrated by removing acetonitrile by rotary evaporation under vacuum (200 mbar) at 45°C and the remaining water displaced by freeze-drying. The 100% MeCN and the acetone wash fractions were also concentrated by rotary evaporation and dried in the fume hood in pre-weighed vials.

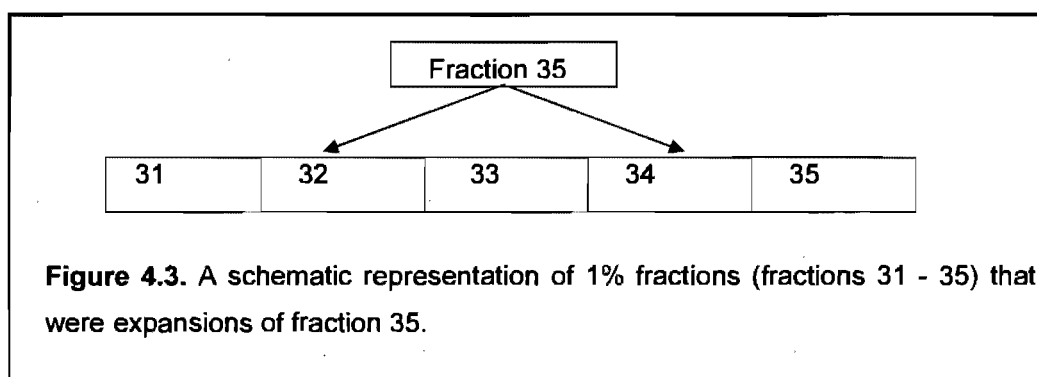
All the fractions were tested for *in vitro* antiparasmodial activity and the results showed that the most active components were being eluted with 30%, 40%

and 50% of MeCN:H<sub>2</sub>O mobile phases. The three fractions were fluffy and cream in colour.

No further work was done on fraction 30 as it was part of an ongoing research in the Division of Pharmacology, University of Cape Town.

Fractions 40 and 50 contained a lot of peaks and had to be purified further to obtain fractions with fewer or single peaks. SPE was then performed using 5% increments of MeCN:H<sub>2</sub>O mobile phases in the range 35% - 55% to expand fraction 40 and 50. The same SPE procedure was followed as before but the 200 ml H<sub>2</sub>O wash was followed by 200 ml of 30% mobile phase to wash off compounds that might be carried over with the 35% mobile phase. Thereafter, compounds were eluted with 200 ml volumes of 35% - 55% mobile phases followed by a 100 ml acetone wash.

The 5% fractions from batch I and batch II were also fluffy and cream in colour. Although these fractions exhibited *in vitro* antiplasmodial activity, their HPLC profiles showed that they contained a number of peaks. The HPLC profiles of fractions 35 - 55 from batch I, which were also similar to those from batch II are shown in Appendix C. The next step was to further purify these fractions on SPE by eluting with 1% mobile phases of MeCN:H<sub>2</sub>O. The first 1% fractions generated, were fractions 31 - 35 which were the expansion of fraction 35 as shown by the diagram in figure 4.4.



**Figure 4.3.** A schematic representation of 1% fractions (fractions 31 - 35) that were expansions of fraction 35.

To generate 1% fractions, the same SPE procedure for generating 10% and 5% fractions was followed. The 1% fractions were only generated from the crude DCM root extract of batch I of the plant roots. In an attempt to optimise separation of 1% fractions and generate pure samples, 300 ml volumes of 31% - 35% mobile phases were used as opposed to 200 ml volumes used for the elution of 10% and 5% SPE fractions. SPE of the crude DCM root extract (4 mg/ml) was carried out using 1% increments of MeCN:H<sub>2</sub>O in the range 31% - 35%. The 200 ml water wash was followed by 300 ml of 30% mobile phase to wash off any compounds that may be carried over with 31% mobile phase. The bound components were eluted with 300 ml of 31% - 35% mobile phases of MeCN:H<sub>2</sub>O followed by 100 ml of acetone.

The fractionation resulted in fluffy and white 1% fractions but as shown by their HPLC profiles (Appendix C), there was no reduction in the number of peaks. Nevertheless, the fractions were tested for *in vitro* antiplasmodial activity against D10, K1, RSA11 and FAC8 strains of *P. falciparum*.

#### **4.2.3. Approaches used to purify 1% fractions**

Several attempts were made to obtain pure compounds from the 1% fractions (31 - 35) using different methods.

##### **4.2.3.1. Method 1: Fractions 31 - 35 generated from fraction 40**

Instead of generating fractions 31 - 35 from the crude DCM root extract, the fractions were generated from fraction 40 since fractions 31 - 39 are the separations of fraction 40. Fraction 40 was reloaded (4 mg/ml) onto the C18 SPE column and eluted with 200 ml volumes of 31% - 35% mobile phases of MeCN:H<sub>2</sub>O. This method did not result in the reduction of peaks.

##### **4.2.3.2. Method 2: Fractions 31 - 35 reloaded onto the SPE column**

More material of fractions 31 - 35 was generated and each fraction was reloaded onto the C18 SPE column, fraction 31 was eluted with 200 ml volumes of 30%, 31% and 32%. Fraction 32 with 200 ml volumes of 31%, 32% and 33%, fraction 33 with 200 ml volumes of 32%, 33% and 34%,

fraction 34 was eluted with 200 ml volumes of 33%, 34% and 35% and fraction 35 was eluted with 200 ml volumes of 34%, 35% and 36%. It was expected that this method will result in the expansion of the peaks but all the samples got stuck onto the column.

#### **4.2.3.3. Method 3: Fraction 31 purified on TLC**

Thin Layer Chromatography (TLC) was used to separate the compounds in fraction 31. The fraction was run on 20 x 20 cm silica gel 60F<sub>254</sub> (0.25 mm thickness) pre-coated aluminium TLC plates (Merck, Darmstadt, Germany) using combinations of mobile phases at different concentrations.

In order to get a mobile phase that will elute as many compounds as possible, it is important that the sample completely dissolve in that mobile phase. Fraction 31 dissolved completely in benzene: acetone (50:50), benzene: ethylacetate (30:70), benzene: acetic acid (60:40), chloroform: methanol (50:50) and ethylacetate: acetate acid (60:40) and was therefore ran in these mobile phases. The sample was loaded onto the plates, 1 cm from the bottom of the plate and let to dry. After drying, the TLC plates were put in developing chambers (24.5 x 7.3 x 24 cm) containing 20 ml of the respective mobile phases and a piece of filter paper to keep the chamber vapourized. The chamber was covered with a lid and the TLC plate developed until the mobile phase was about 1 cm from the top of the plate. A line was drawn to mark the end of the run so as to obtain the R<sub>f</sub> values of the bands. The plates were then viewed under ultra violet (UV) light at 254 nm (short wavelength) and 365 nm (long wavelength).

More bands were observed with the benzene: acetic acid (60:40) mobile phase and the concentrated bands were scraped off and redissolved in 1 ml MeCN. The samples were spun down in a Biofuge 13 centrifuge (Heraeus Sepatech, Osterode, Germany) at 13 000 rpm for 10 minutes. The supernatant was collected, dried under nitrogen and the samples reconstituted in MeCN:H<sub>2</sub>O (50:50).

Although the TLC bands that were scraped off showed fewer peaks on the HPLC, they still needed to be purified further. Therefore, instead of generating fraction 31, run it on the TLC and then on the HPLC to collect individual peaks, the peaks were collected from fraction 31 and 32 since they contained major peaks compared to fractions 33, 34 and 35.

#### **4.2.4. SPE fractionation of the crude DCM root extract from Sterkspruit**

The HPLC profiles of the crude DCM root extracts (cold extraction) of *D. anomala* from Lesotho and Sterkspruit were similar, therefore the crude DCM root extract (4 mg/ml) from Sterkspruit was fractionated to generate fractions 30, 40 and 50. This was to determine whether their HPLC profiles would be similar to those generated from the crude DCM root extract from Lesotho. The SPE fractionation procedure was the same as for the cold DCM root extract from Lesotho but the 200 ml water wash was followed by 200 ml volumes of 30, 40 and 50 mobile phases of MeCN and H<sub>2</sub>O.

### **4.3. High Performance Liquid Chromatography (HPLC)**

High Performance Liquid Chromatography (HPLC) is the most applied separation technique for research and routine analysis purposes. There are two types of HPLC separation, namely isocratic and gradient elution. An isocratic elution, elutes compounds at a single concentration of the mobile phase, while with a gradient elution, compounds are eluted with increasing concentrations of two or more different mobile phases.

The HPLC system consisted of an automatic sample injector, two LC-10AS solvent delivery systems, an SPD –M10A diode array detector, a CBM-10A communications bus module (Shimadzu Corporation, Kyoto, Japan) and a Flexi 486 GV/DX2-66 computer (Sunonwealth Elec, Kaohsiung, Taiwan) with LC workstation software for data registration and analysis (Shimadzu Corporation, Kyoto, Japan). The computer was connected to a Deskjet 670C printer (Hewlett Packard, California, USA). The mobile phase consisted of purified deionised water from a Milli-Q Water System and HPLC grade acetonitrile (Merck, Darmstadt, Germany).

#### **4.3.1. Analytical HPLC conditions**

Stock solutions (1 mg/ml) of the organic extracts and SPE fractions, were made up in 20% MeCN and 80% H<sub>2</sub>O. All the stock solutions were sonicated for 15 minutes and centrifuged on a Biofuge 13 centrifuge at 13 000 rpm for 5 minutes to pellet any undissolved particulate material. The analytical separations of the crude extracts were done on a C18 reverse-phase Phenomenex column (150 x 4.6 mm, 5 µm) (Phenomenex, California, USA), which was connected to a C18 40 µm guard column (Anatech Instruments, Cape Town, South Africa). The gradient used was 20% - 100% MeCN: H<sub>2</sub>O at a flow rate of 1ml/min, the injection volume was 150 µl and the HPLC runs were over 60 minutes and were monitored at 215 nm.

The 10% and 1% SPE fractions were run on a C18 reverse-phase Haisil column (250 x 4.6 mm, 5 µm) (Higgins Analytical, Inc., California, USA), which was connected to a C18 40 µm guard column (Anatech Instruments, Cape Town, South Africa). The gradient was first set from 30% - 100% MeCN: H<sub>2</sub>O then changed to 30% - 70% MeCN: H<sub>2</sub>O and finally to 50% - 70% MeCN: H<sub>2</sub>O over 15 minutes. The HPLC runs were at a flow rate of 1 ml/min and were monitored at 215 nm. The injection volume was 150 µl for the 10% SPE fractions and 250 µl for the 1% SPE fractions.

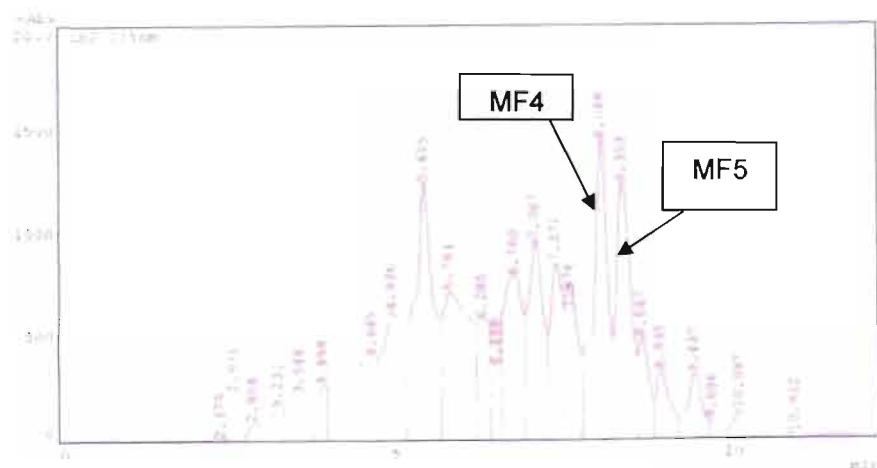
The 5% SPE fractions were ran at a gradient of 30% - 70% MeCN: H<sub>2</sub>O on a C18 reverse-phase Phenomenex column (150 x 4.6 mm, 5 µm)(Phenomenex, California, USA). The column was connected to a C18 40 µm guard column (Anatech Instruments, Cape Town, South Africa). The HPLC runs were monitored at 215 nm over 40 minutes, at a flow rate of 1 ml/min and the injection volume was 150 µl.

##### **4.3.1.1. Collection of peaks from fractions 31 and 32**

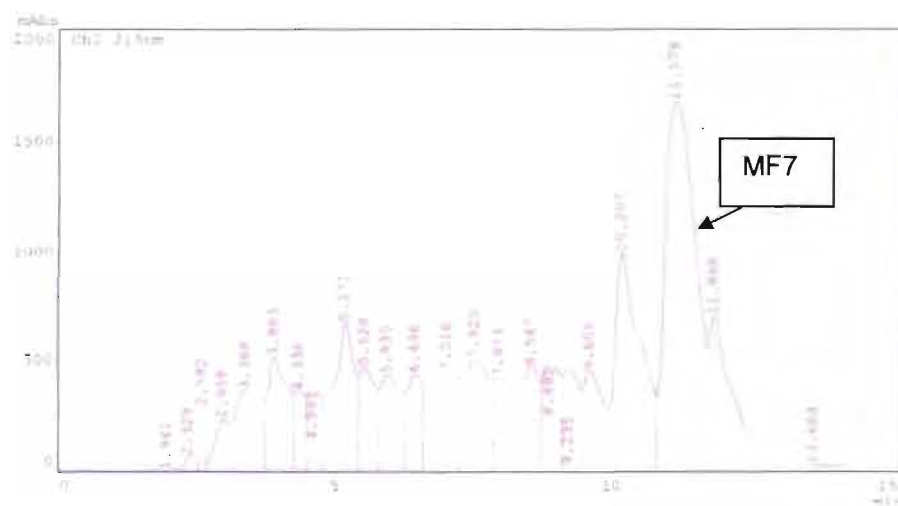
Fractions 31 and 32 (2 mg/ml) were purified on the Haisil analytical column (250 x 4.6 mm) by collecting individual peaks. Peaks MF4 and MF5 were collected from fraction 31 and peak MF7 from fraction 32 as shown in figures 4.4a and 4.4b. The gradient was 50% - 70% MeCN: H<sub>2</sub>O at a flow rate of 1



ml/min and the injection volume was 250  $\mu$ l. The runs were monitored at a wavelength of 215 nm.



**Figure 4.4a.** The HPLC chromatogram of fraction 31 (2 mg/ml) showing peaks MF4 and MF5 eluting at 8.048 minutes and 8.353 minutes, respectively. The fraction was run on a Haisil analytical column (250 x 4.6 mm, 5  $\mu$ m) at a gradient of 50% - 70% MeCN: H<sub>2</sub>O and a flow rate of 1 ml/min. The injection volume was 250  $\mu$ l and the HPLC runs were monitored at a wavelength of 215 nm.



**Figure 4.4b.** The HPLC chromatogram of fraction 32 (2 mg/ml) showing peak MF7 eluting at 11.178 minutes. The fraction was run on a Haisil analytical column (250 x 4.6 mm, 5  $\mu$ m) at a gradient of 50% - 70% MeCN: H<sub>2</sub>O and a flow rate of 1 ml/min. The injection volume was 250  $\mu$ l and the HPLC runs were monitored at a wavelength of 215 nm.

The collected peaks (MF4, MF5 and MF7) were concentrated by evaporating acetonitrile by rotary evaporation under vacuum (200 mbar) at 40°C and displacing the water by freeze-drying. All the samples were fluffy and white in colour.

The MF5 sample changed from a white fluffy form to a brownish oily form after being removed from the freeze-drier. The colour first changed from white to reddish brown then to brownish oily form. This could have been due to exposure to air, which would result in the oxidation of peak MF5 and exposure to moisture, which would result in the shrinking of the compound due to being hygroscopic. To minimise the exposure of MF5 to air and moisture, the flask was covered with parafilm immediately after being removed from the freeze-drier. This approach made a slight improvement as the sample changed after 5 minutes. The collection of peak MF5 was therefore discontinued and the work continued with peaks MF4 and MF7.

#### **4.3.2. Preparative HPLC conditions**

##### **4.3.2.1. Collection of MF4 and MF7 from fractions 40 and 50**

It was discovered that peaks MF4 and MF5 from fraction 31 and peak MF7 from fraction 32 were present in fraction 40 and only peak MF7 was present in fraction 50. There was also more material generated for fractions 40 and 50 as compared to 1% fractions. Therefore, peaks MF4 and MF7 were both collected by semi-preparative reversed-phase HPLC from fraction 40 and only peak MF7 was collected from fraction 50 (Chapter 7, figures 7.2a and 7.2b, p. 129). Fractions 40 and 50 (5 mg/ml) were loaded on a C18 Haisil 100 reverse-phase semi-preparative column (250 x 10 mm, 5 µm) (Higgins Analytical, Inc., California, USA), which was connected to a C18 40 µm guard column (Anatech Instruments, Cape Town, South Africa). A gradient elution of 50% - 70% MeCN: H<sub>2</sub>O was used at a flow rate of 3 ml/min and the injection volume was 260 µl. The detection wavelength was set at 215 nm.

#### **4.4. Separation of the components in peak MF7**

Although peak MF7 eluted as a single peak, the NMR revealed that it was impure and the Mass spectrometry showed that there were a few components present in peak MF7 (Chapter 7, figures 7.4a and 7.4b, p. 130-131). Three approaches were tried in order to separate these components in peak MF7.

##### **4.4.1. Recrystallization**

A small amount of MF7 (1 mg) was dissolved in petroleum ether (50  $\mu$ l) and a few drops of ethylacetate were added until the solution was cloudy. The cloudy solution of MF7 was stored in a 4°C fridge for two weeks. This was to determine whether the major component of the mixture will crystallize. After two weeks, white crystals had formed. The crystals were collected by vacuum filtration in a Büchner funnel lined Whatman no. 6 filter paper (Whatman Limited, Maidstone, England). The filter paper was first moistened with hexane. The crystals were rinsed with hexane, under suction and dried in the fume hood. The recrystallization was unsuccessful as the Mass spectrometry analysis showed that all the components were present in the crystals.

##### **4.4.2. C16 amide column**

A less hydrophobic analytical column with amide groups, a C16 amide column (Supelco, Sigma-Aldrich, St Louis, Missouri, USA) was used to separate the three peaks in MF7 using gradient and isocratic elutions of MeCN and H<sub>2</sub>O but no separations of the peaks were achieved.

##### **4.4.3. Isocratic elution of fraction 40**

Fraction 40 (4.5 mg/ml) was run isocratically at 50%, 60% and 70% MeCN in H<sub>2</sub>O on a C18 Haisil reverse-phase semi-preparative column, at a flow rate of 2.5 ml/min and a detection wavelength of 215 nm. The injection volume was 250  $\mu$ l.

The MF7 peak separated into two peaks with the 60% isocratic elution, though the separation was not good. The injection volume was reduced to 150  $\mu$ l and

the MF7 peak separated into three peaks although the resolution was not good. The three peaks of MF7 were designated MF7A, MF7B and MF7C, of which the major peak was MF7A. The same HPLC conditions used to elute fraction 40 were also used for the elution of fraction 50. MF7A was easier to collect since it was present in higher concentrations as compared to MF7B and MF7C.

The procedure, therefore, of isolating compounds MF4 and MF7A from fraction 40 and MF7A from fraction 50, was by loading 4.5 mg/ml of the fractions on a C18 Haisil 100 semi-preparative column (250 x 10 mm, 5 µm) and eluting the fractions with an isocratic run of 60% MeCN: H<sub>2</sub>O at a flow rate of 2.0 ml/min. The injection volume was 150 µl and the detection wavelength was set at 215 nm.

#### **4.5. Purification of Compounds MF4 and MF7A**

Compounds MF4 and MF7A (2 mg/ml) were purified on a Haisil analytical columns (250 x 4.6 mm, 5 µm for MF7A and 150 x 4.6 mm, 5 µm for MF4) at an isocratic elution of 60% MeCN: H<sub>2</sub>O, the flow rate was 1 ml/min and the injection volume was 100 µl. The compounds were monitored at a wavelength of 215 nm. The compounds were concentrated by rotary evaporating acetonitrile under vacuum (200 mbar) at 40 °C and displacing the water by freeze-drying.

#### **4.6. *In vitro* Antiplasmodial Activity**

##### **4.6.1. Reagents**

Chloroquine diphosphate, nitro blue tetrazolium, phenazine ethosulphate, APAD, D-sorbitol, glucose, hypoxanthine, HEPES buffer, Tris buffer, L-Lactate and NaHCO<sub>3</sub> were obtained from Sigma-Aldrich, Kempton Park, South Africa. Triton X-100, Giemsa stain and methanol were obtained from Merck, Modderfontein, South Africa. RPMI 1640 medium was obtained from Highveld Biological, Johannesburg, South Africa and gentamycin from Micro Healthcare, Bethlehem, Bloemfontein, South Africa. Albumax II was obtained

from Gibco-BRL, Auckland, New Zealand. The blood was obtained from the blood bank in Groote Schuur Hospital, Cape Town, South Africa.

#### **4.6.2. *In vitro* culture of *Plasmodium falciparum***

The four strains of *Plasmodium falciparum*, a chloroquine-sensitive (CQS) strain (D10) and chloroquine-resistant (CQR) strains (RSA11, K1 and FAC8) were cultured continuously according to the methods of Trager and Jensen, 1976. The parasite cultures were maintained *in vitro* in O<sup>+</sup> human red blood cells suspended in filter-sterilized complete medium. The complete medium contained 10.4 g/L RPMI 1640 medium with glutamine, supplemented with 40 µg/ml gentamycin, 0.088 g/L hypoxanthine, 4 g/L glucose, 6 g/L HEPES buffer, 5 g/L albumax II and 8.4 ml of 5% NaHCO<sub>3</sub> per 200 ml medium, which was added before use.

The parasite cultures were kept in tissue culture flasks (Greiner Bio-One, Frickenhausen, Germany) in an atmosphere of filtered gas containing 4% CO<sub>2</sub>, 3% O<sub>2</sub> and 93% N<sub>2</sub> and were incubated at 37°C in a CO<sub>2</sub> Water - Jacketed (IR Autoflow) incubator (Nuair, Minnesota USA). The culture medium was changed daily by transferring the cultures from the flask into a 50 ml tube (Greiner Bio-One, Frickenhausen, Germany) and centrifuging at 750 rpm for 5 minutes in an eppendorf centrifuge 5804 (Eppendorf centrifuge, Hamburg, Germany). The medium was aspirated and a thin blood smear was prepared on a slide, fixed with methanol and stained with Giemsa stain (10% in PBS). The slide was examined under the microscope to monitor the parasitaemia. Fresh blood was added to maintain a 5% parasitaemia and 4% haematocrit. An aliquot of 45 ml complete medium (warmed at 37 °C) was added to the parasite cultures, put back in the culture flask, gassed for ± 1 minute and put back into the incubator. The parasitemia was determined by examining a minimum of 500 red blood cells for infection by parasites. The parasitemia was calculated as follows:

$$\% \text{ Parasitemia} = \frac{\text{number of infected red blood cells}}{\text{total number of red blood cells}}$$

#### **4.6.3. Parasite synchronization**

The parasites cultures were synchronized before each experiment as described by Lambros and Vanderberg, 1979. The cultures were synchronised by D-sorbitol lysis (5% w/v) when parasites were in the ring stage. Only red blood cells infected with mature trophozoites and schizonts are lysed, thereby killing the parasites. Red blood cells infected with ring forms or young trophozoites survive the treatment.

#### **4.6.4. Parasite lactate dehydrogenase (pLDH) Assay**

Parasite survival was measured by parasite lactate dehydrogenase (pLDH) assay as described by Makler, 1993. This enzymatic assay involves the parasite lactate dehydrogenase, which is distinguishable from the host lactate dehydrogenase. Lactate dehydrogenase is an enzyme found in all the cells and catalyses the formation of pyruvate from lactate reducing a co-enzyme NAD (nicotinamide adenine dinucleotide) to NADH. In parasites the NAD analogue APAD (3-acetylpyridine adenine nucleotide) is reduced to APADH and upon this reduction the yellow NBT/PES (nitro blue tetrazolium + phenazine ethosulphate) is converted to purple formazan crystals. The formation of these crystals indicates the pLDH activity and therefore the survival of parasites.

#### **4.6.5. Preparation of stock solutions for the Antiplasmodial Assay**

Stock solutions of the test samples were prepared in sterile eppendorfs at a concentration of 2 mg/ml. All test samples (2 mg), except for water extracts, were dissolved in 100 µl acetonitrile and sonicated for 15 minutes. The stock solution was made up to 2 mg/ml by adding 900 µl of Millipore water and sonicated for a further 15 minutes. The water extracts (2 mg) were dissolved

in 1 ml Millipore water and sonicated for 30 minutes. Sonication ensures that any bacteria or fungi that may be present in the stock solution are killed by breaking down their cell walls. The solvent control was prepared by adding 100  $\mu$ l of acetonitrile to 900  $\mu$ l of Millipore water and sonicating for 30 minutes. This was to confirm that a final concentration of 0.1% acetonitrile exhibited no antiplasmodial activity. The antimalarial drug, chloroquine (2 mg/ml in Millipore water) was prepared as a positive control. Dilutions (1 in 10) of test samples were prepared in culture medium to make up a starting concentration of 200  $\mu$ g/ml. For chloroquine, the starting concentration was 2000 ng/ml.

#### **4.6.6. Antiplasmodial Assay**

Assays were performed when parasite cultures were in trophozoite stages and were done in duplicates in 96-well culture plates on two separate experiments.

In a sterile 96-well culture plate (Greiner Bio-One, Frickenhausen, Germany), arranged in 8 rows (A – H) and 12 columns (1 – 12), the complete medium (100  $\mu$ l) was added in all the wells except those in column 3. To the wells in column 3, 200  $\mu$ l of 200  $\mu$ g/ml test samples was added in duplicates. Two-fold serial dilutions of the test samples were made across the plate by removing 100  $\mu$ l from the wells in column 3, transferring it to the wells in column 4 and resuspending. This was repeated up to column 12 and the remaining 100  $\mu$ l was discarded. The blank was prepared by adding 100  $\mu$ l of red blood cells with 2% haematocrit to column 1. To the remaining columns, 100  $\mu$ l of 2% parasitised red blood cells was added. Column 2 served as the control (medium and parasitised red blood cells only). The final concentrations of the test samples ranged from 100  $\mu$ g/ml to 0.195  $\mu$ g/ml and those of chloroquine ranged from 1000 ng/ml to 1.95 ng/ml. The parasites were exposed to a final solvent concentration of 0.1% acetonitrile and the final haematocrit was 1%. The plate was covered with a sterile lid, placed in a chamber and the chamber gassed with filtered gas containing 3% O<sub>2</sub>, 4% CO<sub>2</sub> and 93% N<sub>2</sub>. The chamber was left for 48 hours at 37°C in a CO<sub>2</sub> Water - Jacketed (IR Autoflow)

incubator (Nuaire, Minnesota USA). Figure 4.5 shows the plate set up for the experiment.

	1	2	3	4	5	6	7	8	9	10	11	12
A		C				test	sample 1 (CQ)					
B	B	O										
C	L	N				test	sample 2					
D	A	T	100	50	25	12.5	6.25	3.125	1.562	0.781	0.391	0.195
E	N	R				test	sample 3					
F	K	O										
G		L				test	sample 4					
H												

**Figure 4.5.** A plate set up for the antiplasmodial assay.

Rows (A – H) contained 4 test samples done in duplicates as shown by the four different colours. Column 1 was a blank (medium and red blood cells) and column 2 a control (medium and parasites). Columns 3 to 12 contained decreasing concentrations of the test samples from a 100 µg/ml to 0.195 µg/ml.

After 48 hours, all the wells were re-suspended. In a separate 96-well culture plate, a 100 µl aliquot of Malstat reagent (4g L-Lactate, 1.32g Tris buffer, 400 µl Triton X-100 and 22 mg APAD in 200 ml Millipore water, pH = 9 with HCl) was added to all the wells, followed by a 15 µl aliquot of the cell suspension to the respective wells. Thereafter, 25 µl of nitro blue tetrazolium/phenazine ethosulphate (NBT/PES) (160 mg NBT and 8 mg PES in 100 ml Millipore water) was added to all the wells and the plate was left to develop in the dark, as NBT/PES is light sensitive. The plate was read at 620 nm on an anthos htll plate reader (Anthos Labtec Instruments, Eugendorf, Austria).

The antiplasmodial activity of test samples was expressed by inhibitory concentrations that inhibit 50% growth of parasites ( $IC_{50}$ ). The inhibitory effects of the test samples were expressed as percentage parasite survival of the untreated parasitised red blood cells. The untreated parasitised red blood cells were referred to as 100% growth. Sigmoidal dose response curves were obtained by plotting the percentage survival against the logarithm of



concentrations using GraphPad Prism 4 software (GraphPad, California, USA). The  $IC_{50}$  values were then calculated by non-linear regression analysis processed on dose-response curves.

#### **4.7. *In vitro* Cytotoxicity Assay**

The *in vitro* cytotoxicity of the test samples was estimated on Chinese Hamster Ovarian (CHO) cells and the cell growth was measured by MTT assay.

##### **4.7.1. Reagents**

MTT, HAMS F-12 were obtained from Sigma-Aldrich, Kempton Park, South Africa, foetal calf serum (FCS), trypsin and DMEM were obtained from Highveld Biological, Johannesburg, South Africa. DMSO was obtained from Merck.

##### **4.7.2. MTT Assay**

The MTT assay is a colorimetric assay that utilizes the water-soluble yellow coloured MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide], a tetrazolium salt, which upon reduction is converted to a water-insoluble purple coloured formazan product [Sieuwerts et al., 1995]. This assay measures the metabolic viability of cells *in vitro*, therefore it uses only viable cells. The metabolic viability is determined by measuring the activity of various dehydrogenase enzymes. This is supported by the fact that the tetrazolium ring is cleaved in functional mitochondria [Mosmann, 1983]. The amount of product formed is proportional to the metabolic activity and the number of cells in the test sample [Sieuwerts et al., 1995].

The CHO cells were kept in complete medium, in tissue culture flasks with filter caps (Greiner Bio-One, Frickenhausen, Germany). The complete medium consisted of filter-sterilized 10% heat-inactivated (56°C for 30 minutes) foetal calf serum (FCS), 45% Dulbeccos Eagles Modified Medium (DMEM) (pH = 7.1) and 45% HAMS F-12 (pH = 7.1). DMEM and HAMS F-12

contained 500 µl/L of gentamycin to kill any bacteria that might contaminate the medium.

#### **4.7.3. Sub-culturing cells**

The cells were sub-cultured when they had reached confluency (when the entire flask is covered with elongated cells). The medium was removed from the flask and the cells rinsed twice with 10 ml sterile phosphate buffered saline (PBS) to remove FCS. An aliquot of 5 ml trypsin, warmed at 37°C was added to the cells and the flask incubated for 2 minutes. Trypsin detaches the cells from the flask. The flask was agitated slightly to lift cells off the flask and 5 ml of complete medium added to inhibit trypsin reaction, which if not stopped in time, will digest the cells. The cell cultures were then transferred to 15 ml cellstar tubes (Greiner Bio-One, Frickenhausen, Germany) and centrifuged at 750 rpm for 5 minutes. After centrifugation, the medium was poured off and the cell pellet resuspended in 5 ml of complete medium. A 1 ml aliquot of the cell suspension, supplemented with 9 ml complete medium was transferred to a new flask and incubated at 37°C in 5% CO<sub>2</sub>-95% air humidified atmosphere in a CO<sub>2</sub> Air - Jacketed (DHD Autoflow) incubator (Nuaire, Minnesota USA).

The medium was changed every 2-3 days when cells had not reached confluency and when the medium was slightly yellow that is when the pH has dropped to acidic. A 5 ml aliquot of the acidic medium was removed and replaced with 5 ml fresh complete medium and the flask placed back into the incubator.

#### **4.7.4. Preparation of stock solutions for the cytotoxicity assay**

Stock solutions of the test samples were prepared the day before the experiment was carried out otherwise they were stored at -20°C. Test samples were prepared at a concentration of 2 mg/ml by dissolving the test samples in 100 µl acetonitrile and sonicating for 15 minutes. This was made up to 1 ml by adding 900 µl of Millipore water and sonicating the stock solution for a further 15 minutes. On the day of the experiment, six 10-fold serial

dilutions of the stock solution were prepared in complete medium from 200 µg/ml to 0.002 µg/ml.

#### 4.7.5. Plating cells

For plating cells, the sub-culturing method was followed. The cell pellet was resuspended in 5 ml of complete medium. A 20 µl aliquot of the resuspended cells was mixed with 20 µl of the crystal violet solution in an eppendorf to stain the cells for counting. A 20 µl aliquot of this was removed, placed on a counting chamber of the hemacytometer (Sigma-Aldrich, Kempton Park, South Africa) and covered with a coverslip. Cells were counted from the central grid (5 x 5 squares). To ensure that the cells were viable and in their logarithmic stage,  $1 \times 10^4$  cells were seeded at a density of approximately  $1 \times 10^4$  cells/ml per well. The following calculation was used:

$$\begin{aligned} \text{Number of cells/cubic millimeter (mm}^3\text{)} &= \\ \text{Average number of cells counted} \times 25 \times 2 \times 10^4 \text{ cells /ml} \end{aligned}$$

Where 25 = area of the central grid (5 x 5)  
2 = dilution factor  
 $\text{mm}^3 = \text{ml}$

The initial stock solution of the cells was made up to  $10^5$  cells/ml, so that after addition of the medium in the wells the final density will be  $1 \times 10^4$  cells /ml. Sterile 96-well microtitre culture plates (Greiner Bio-One, Frickenhausen Germany) arranged in 8 rows (A – H) and 12 columns (1 – 12) were used for plating cells. The plate was turned around so that rows A – H were now columns H – A and columns 1 – 12 were now rows 1 –12 (figure 4.6). To the wells used as blanks (column H), 200 µl of complete medium was added. To the rest of the wells 100 µl of cell suspension was added together with 100 µl of complete medium. The cells were resuspended after adding to every two rows since they settled down quickly. Column G was used as the control, with medium and cells only. The plate was incubated for 24 hours at 37°C in 5% CO<sub>2</sub> - 95% air humidified atmosphere to allow the cells to attach to the bottom of the wells.

#### 4.7.6. Cytotoxicity Assay

After 24 hours, all the wells were checked to see if the cells have attached. The medium was then aspirated from all the wells leaving a thin film of complete medium covering the cells to avoid aspirating the cells as well. Aliquots of 100  $\mu$ l of the test samples were added to the corresponding wells in triplicates (column F to column A) at concentrations from (200  $\mu$ g/ml to 0.002  $\mu$ g/ml), followed by 100  $\mu$ l of complete medium. The final concentrations therefore, ranged from 100  $\mu$ g/ml to 0.001  $\mu$ g/ml. To the wells used as blanks (column H, complete medium only) and control (column G, cells and complete medium only), 200  $\mu$ l of complete medium was added. The plate set up is shown in figure 4.6.

	H	G	F	E	D	C	B	A
1			100	10	1	0.1	0.01	0.001
2				Test	sample 1(EMT)			
3		C						
4	B	O						
5	L	N		test	sample	2		
6	A	T						
7	N	R						
8	K	O		test	sample	3		
9		L						
10								
11				test	sample	4		
12								

**Figure 4.6.** A plate set up for the cytotoxicity assay. EMT = emetine.

The control was referred to as 100% cell viability. Emetine served as a positive control and acetonitrile (0.1%) as a negative control. The plate was covered with a lid and incubated for 48 hours at 37°C in 5% CO<sub>2</sub> -95% air humified atmosphere. The assays were done on two separate experiments.

After 48 hours, cell viability was measured by MTT assay. To each well, 25  $\mu$ l of MTT (5 mg/ml in Millipore water) was added (protected from light since it is sensitive to light) and the plate was incubated for a further 4 hours at 37°C for the MTT cleavage. After incubation, the plate was centrifuged in an eppendorf centrifuge for 10 minutes at 200 rpm and the medium was removed by aspiration. An aliquot of 100  $\mu$ l of dimethyl sulfoxide (DMSO) was added to each well and the plate shaken vigorously on a vortex-genie microplate shaker, (Scientific Industries, New York, USA) at a low speed for 5 minutes. This was to ensure that the formed purple formazan crystals were dissolved. The optical density of each well was measured at 540 nm on an anthos htll microplate reader.

The cytotoxicity effects of test samples were expressed as percentage cell viability of the drug-free cells. Sigmoidal curves were obtained by plotting percentage cell viability against logarithmic concentrations and the IC<sub>50</sub> values were calculated by non-linear regression analysis processed on dose-response curves. GraphPad Prism 4 software was used to plot all the graphs.

#### **4.7.7. Selectivity Index**

The selectivity index is obtained to determine whether a compound or extract is cytotoxic or antiplasmodial. The selectivity index of the test samples were determined by calculating the ratio of cytotoxicity to antiplasmodial activity, with the units used being the same. The higher the selectivity index, the less toxic the test sample.

$$\text{Selectivity Index} = \text{Cytotoxicity IC}_{50} / \text{Antiplasmodial IC}_{50}$$

#### **4.7.8. Resistance Index**

The resistance index is used to give an indication of the relative activity of the compounds in drug-resistant and sensitive strains.

$$\text{Resistance Index} = K1 \text{ IC}_{50} / D10 \text{ IC}_{50}$$

#### 4.8. Spectroscopy for Compounds MF4 and MF7A

$^1\text{H}$  and  $^{13}\text{C}$  NMR spectral analysis were recorded on a Varian <sup>UNITY</sup> Inova 600 spectrometer (Varian Inc., California, USA) in  $\text{CDCl}_3$  at 25 °C with TMS as internal standard. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded at frequencies of 600 MHz and 150 MHz, respectively. The complete assignment for proton and carbon atoms was enabled by the extensive 2D NMR analysis which included  $^1\text{H}$  -  $^1\text{H}$  COSY, NOESY, HMQC and HSQC experiments. The low resolution mass spectra were run on API 2000 LC/MS/MS mass spectrometer (Applied Biosystems, California, USA). The HRMS (high resolution mass spectra) were run on a Waters API Q-TOF Ultima (Waters, Milford, USA). The conditions for the Electrospray ionisation (ESI) probe were a desolvation temperature of 350 °C and desolvation gas was at 400 L/h, the capillary voltage was 3.5 kV with a temperature of 100 °C, cone voltage was 35 V and cone gas was at 50 L/h. Mass range scanned was between 200 – 1900 atomic mass units (amu).

## **CHAPTER 5**

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### **EVALUATION OF THE CRUDE EXTRACTS OF *Dicoma anomala* FOR ANTIPLASMODIAL ACTIVITY**

## 5.1. INTRODUCTION

The roots of *D. anomala*, collected from Lesotho, Sterkspruit, Limpopo and Reitz were evaluated for antiplasmodial activity *in vitro* against the D10 strain of *P. falciparum*. The leaves and twigs (except for the plant collected in Lesotho) were also screened for *in vitro* antiplasmodial activity.

The roots of *D. anomala* (Sond.) from Sterkspruit, Limpopo and Reitz were collected in October 2003 and those from Lesotho were collected in December 1996 (batch I) and October 2003 (batch II).

## 5.2. METHODS

Cold and hot extracts of the roots of *D. anomala* were prepared with dichloromethane (DCM) and water (H<sub>2</sub>O), while the cold extracts of the leaves and twigs were prepared with DCM only. In addition, cold extractions of the roots of *D. anomala* from Lesotho were prepared with methanol (MeOH), hexane, ethylacetate, petroleum spirit and chloroform. The cold extractions were prepared using the mechanical shaker and the hot extractions were prepared from the soxhlet extractor and the hotplate.

The extracts which showed antiplasmodial activity against the CQS strain, D10, were screened for cytotoxicity on the Chinese Hamster Ovarian (CHO) cells. Details of the methods used are in Chapter 4.

## 5.3. RESULTS AND DISCUSSION

The antiplasmodial activity and the cytotoxicity of the extracts were determined at 50% inhibitory concentration (IC<sub>50</sub>). The antiplasmodial activity, the cytotoxicity and the selectivity indices of the extracts from the four locations are shown in table 5.1. For antiplasmodial activity, the test samples with IC<sub>50</sub> ≤ 10 µg/ml were considered active and worthy of further evaluation.



The DCM leaf and twig extracts from Sterkspruit, Limpopo and Reitz showed antiparasmodial activity against the D10 strain of *P. falciparum* ( $3.14 \pm 0.12 \mu\text{g/ml}$ ,  $1.67 \pm 0.22 \mu\text{g/ml}$ ,  $0.68 \pm 0.025 \mu\text{g/ml}$ , respectively). The two extracts (Limpopo =  $4.58 \pm 1.02 \mu\text{g/ml}$  and Reitz =  $3.85 \pm 0.13 \mu\text{g/ml}$ ) showed a high cytotoxicity but their selectivity indices (2.78 and 5.66, respectively) showed that the antiparasmodial activity was not due to the high cytotoxicity. The DCM leaf and twig extract from Sterkspruit would therefore be the good candidate extract for the isolation of antiparasmodial activity because it showed less cytotoxicity ( $31.12 \pm 0.22 \mu\text{g/ml}$ ) and high selectivity index (9.91) compared to the other two extracts. These extracts contained fewer peaks as shown by their HPLC profiles (Appendix A). The results confirm the traditional use of the leaf and twig extracts of *D. anomala* for the treatment of malaria and fever [Neuwinger, 2000].

**Table 5.1.** The antiparasmodial activity of the extracts of *D. anomala* on the D10 strain of *P. falciparum* and the cytotoxicity of the antiparasmodial active extracts on the CHO cells.

Extract	Collection Location	IC <sub>50</sub> mean $\pm$ S.D. ( $\mu\text{g/ml}$ ) D10 strain	IC <sub>50</sub> mean $\pm$ S.D. ( $\mu\text{g/ml}$ ) CHO cells	Selectivity Index (SI)
<b>Leaves and twigs</b>				
Cold DCM	Sterkspruit	$3.14 \pm 0.12$	$31.12 \pm 0.22$	9.91
	Limpopo	$1.67 \pm 0.22$	$4.58 \pm 1.02$	2.78
	Reitz	$0.68 \pm 0.025$	$3.85 \pm 0.13$	5.66
<b>Roots</b>				
Cold DCM	Lesotho (BI)*	$2.48 \pm 0.005$	-	-
	Lesotho (BII)*	$1.55 \pm 0.07$	$9.82 \pm 0.25$	6.33
	Sterkspruit	$1.02 \pm 0.095$	$6.67 \pm 0.93$	6.54
	Limpopo	$1.39 \pm 0.20$	$5.34 \pm 1.18$	3.84
	Reitz	$0.48 \pm 0.045$	$3.50 \pm 0.39$	7.29
<b>Hot DCM</b>				
	Lesotho	$1.44 \pm 0.27$	$9.43 \pm 0.92$	6.55
	Sterkspruit	$1.07 \pm 0.09$	$4.80 \pm 0.24$	4.48
	Limpopo	$0.42 \pm 0.04$	$2.90 \pm 0.31$	6.90
	Reitz	$1.25 \pm 0.15$	$2.84 \pm 0.055$	2.27

table 5.1. cont.

Extract	Collection Location	IC <sub>50</sub> mean ± S.D. (µg/ml) D10 strain	IC <sub>50</sub> mean ± S.D. (µg/ml) CHO cells	Selectivity Index (SI)
Cold H <sub>2</sub> O	Lesotho	23.86 ± 0.90	-	-
	Sterkspruit	21.85 ± 1.35	-	-
	Limpopo	13.92 ± 0.52	-	-
	Reitz	29.76 ± 2.90	-	-
Hot H <sub>2</sub> O (soxhlet) 6 hrs	Lesotho	> 100	-	-
	Sterkspruit	> 100	-	-
	Limpopo	61.15 ± 3.25	-	-
	Reitz	66.72 ± 6.74	-	-
Hot H <sub>2</sub> O (hotplate) 6 hrs	Lesotho	69.46 ± 2.16	-	-
	Sterkspruit	43.42 ± 1.44	-	-
	Limpopo	17.06 ± 1.22	-	-
	Reitz	48.74 ± 2.08	-	-
Hot H <sub>2</sub> O (hotplate) 2 hrs	Lesotho	24.16 ± 0.16	-	-
	Sterkspruit	19.54 ± 0.045	-	-
	Limpopo	8.44 ± 0.25	87.06 ± 6.70	10.32

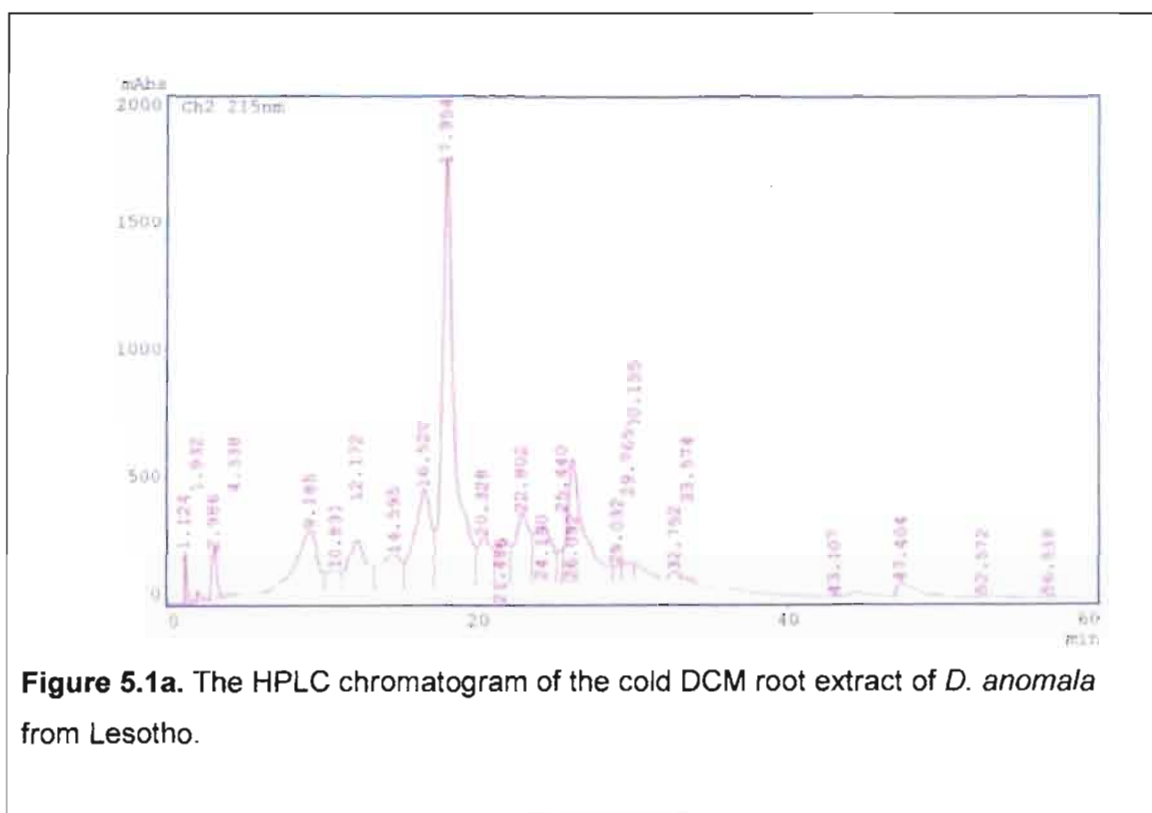
\* BI and BII = batch I and II of *D. anomala* roots from Lesotho.

~ Not done

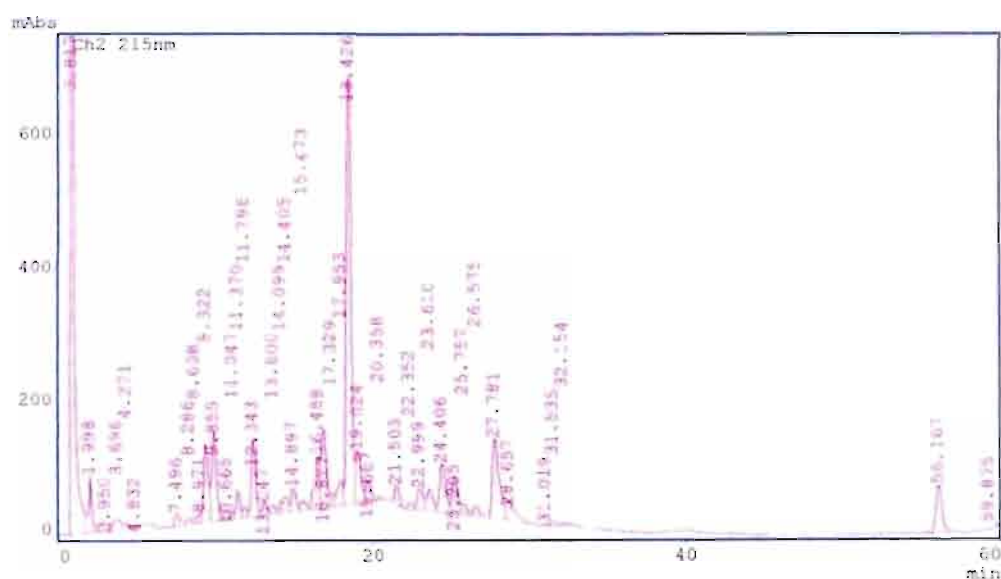
The cold and hot DCM root extracts of *D. anomala* from Lesotho, Sterkspruit, Limpopo and Reitz also showed antiplasmodial activity (IC<sub>50</sub> ≤ 1.55 µg/ml) against the D10 strain of *P. falciparum*. The extracts were shown to be cytotoxic on the CHO cells (IC<sub>50</sub> < 10 µg/ml) but their selectivity indices indicated that they are more selective towards the parasites than to the cells (table 5.1).

The cold and hot DCM root extracts from Lesotho (1.55 ± 0.07 µg/ml and 1.44 ± 0.27 µg/ml, respectively) had similar antiplasmodial activity and this indicated that heat had no effect on the compounds, and similarly with cold and hot DCM extracts from Sterkspruit (1.02 ± 0.095 µg/ml and 1.07 ± 0.09 µg/ml, respectively). There was similarity in the HPLC profiles of the cold and hot DCM

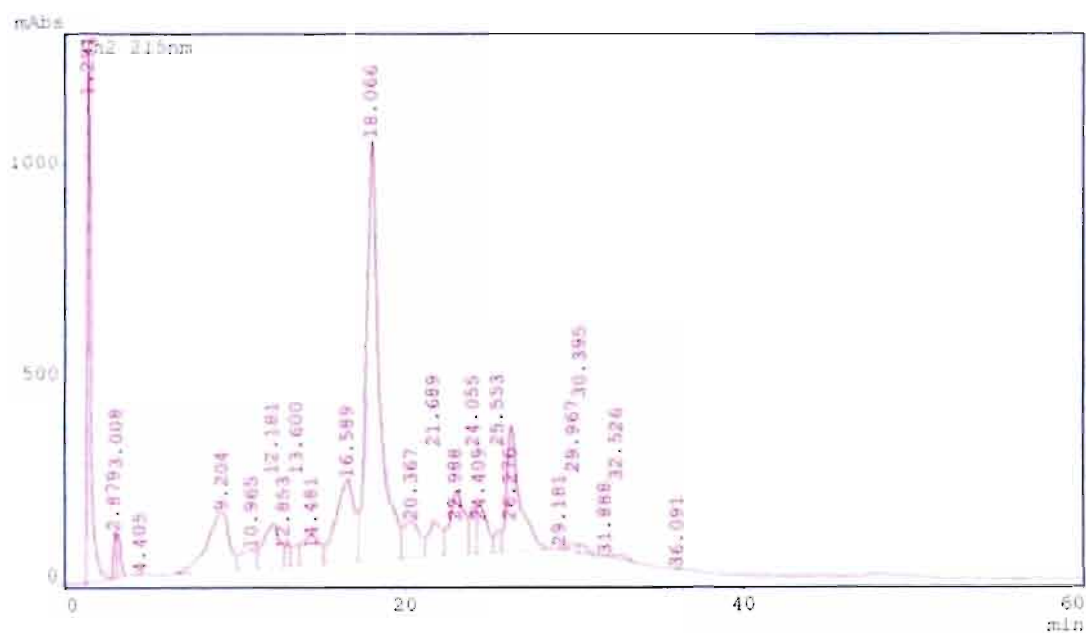
extracts from Lesotho and Sterkspruit (figures 5.1a, 5.1b, 5.2a and 5.2b), which confirmed that no compounds were lost or destroyed during the hot extraction of the roots with DCM. The HPLC profiles of the cold DCM root extracts from Limpopo and Reitz were different to those from Lesotho and Sterkspruit (Appendix A).



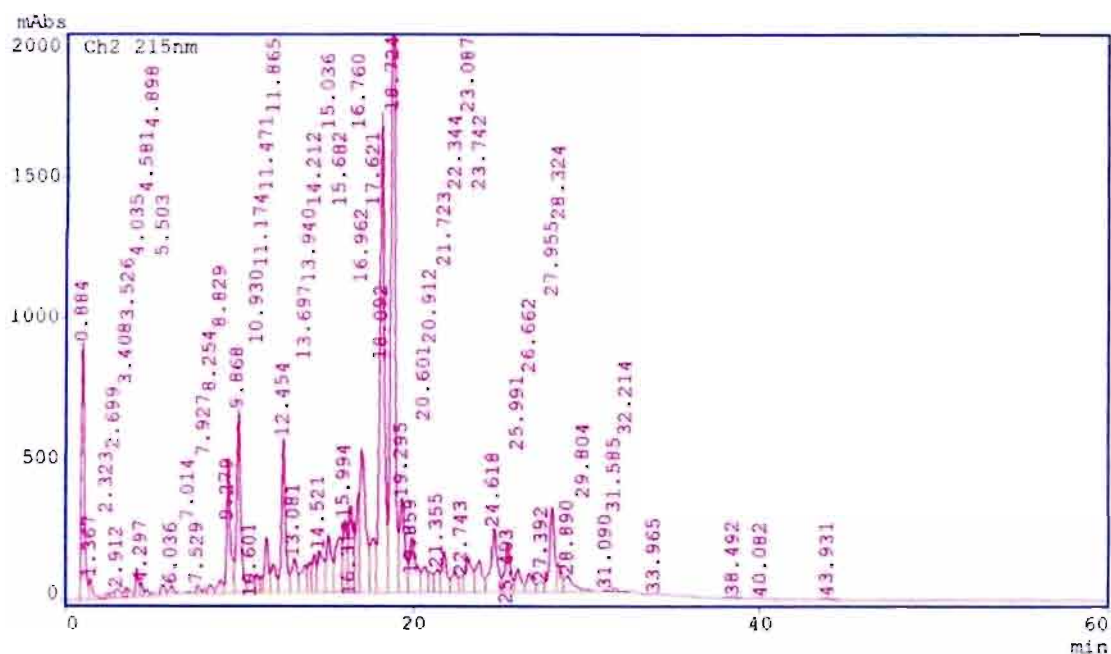
**Figure 5.1a.** The HPLC chromatogram of the cold DCM root extract of *D. anomala* from Lesotho.



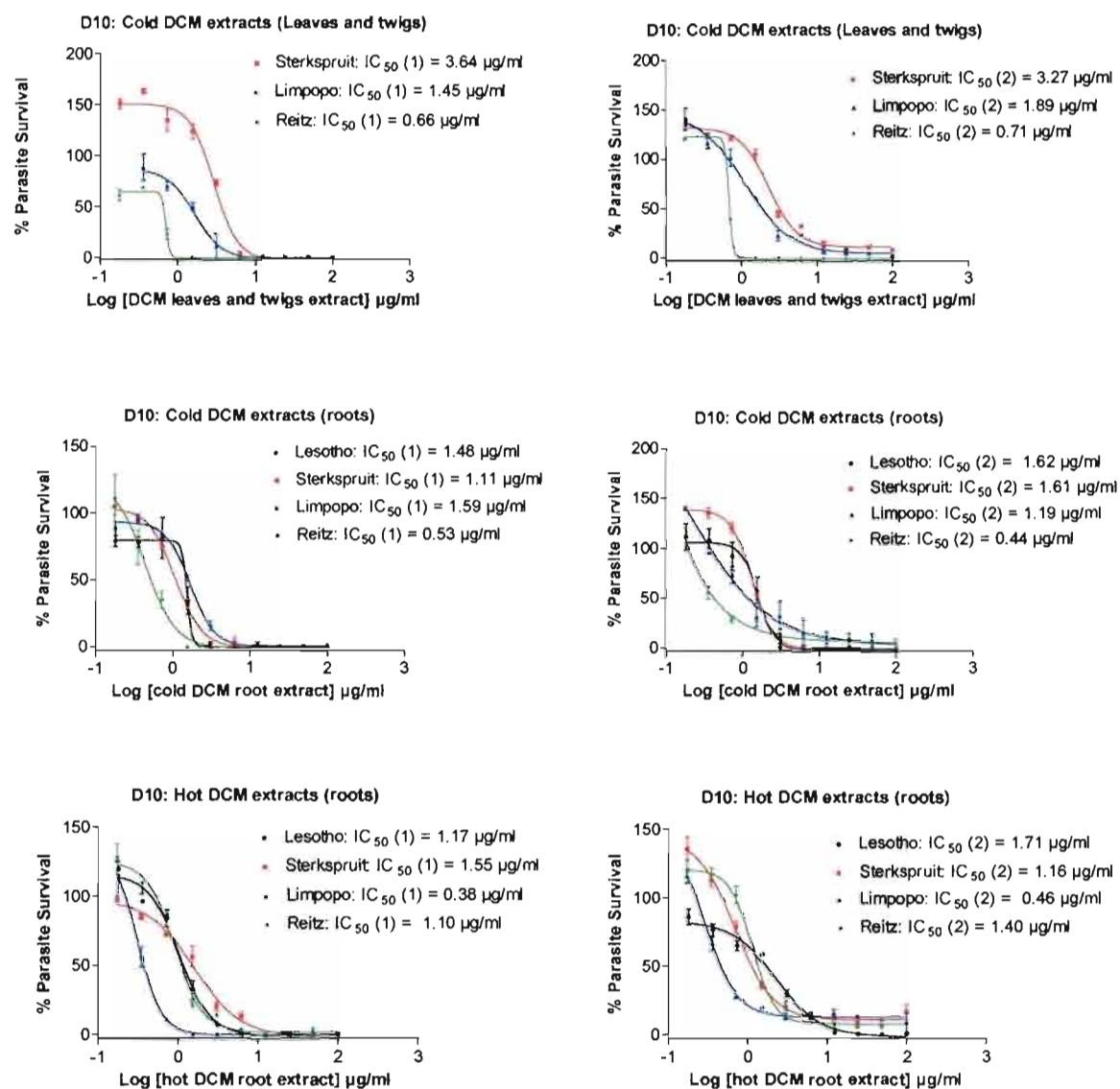
**Figure 5.1b.** The HPLC chromatogram of the hot DCM root extract of *D. anomala* from Lesotho.



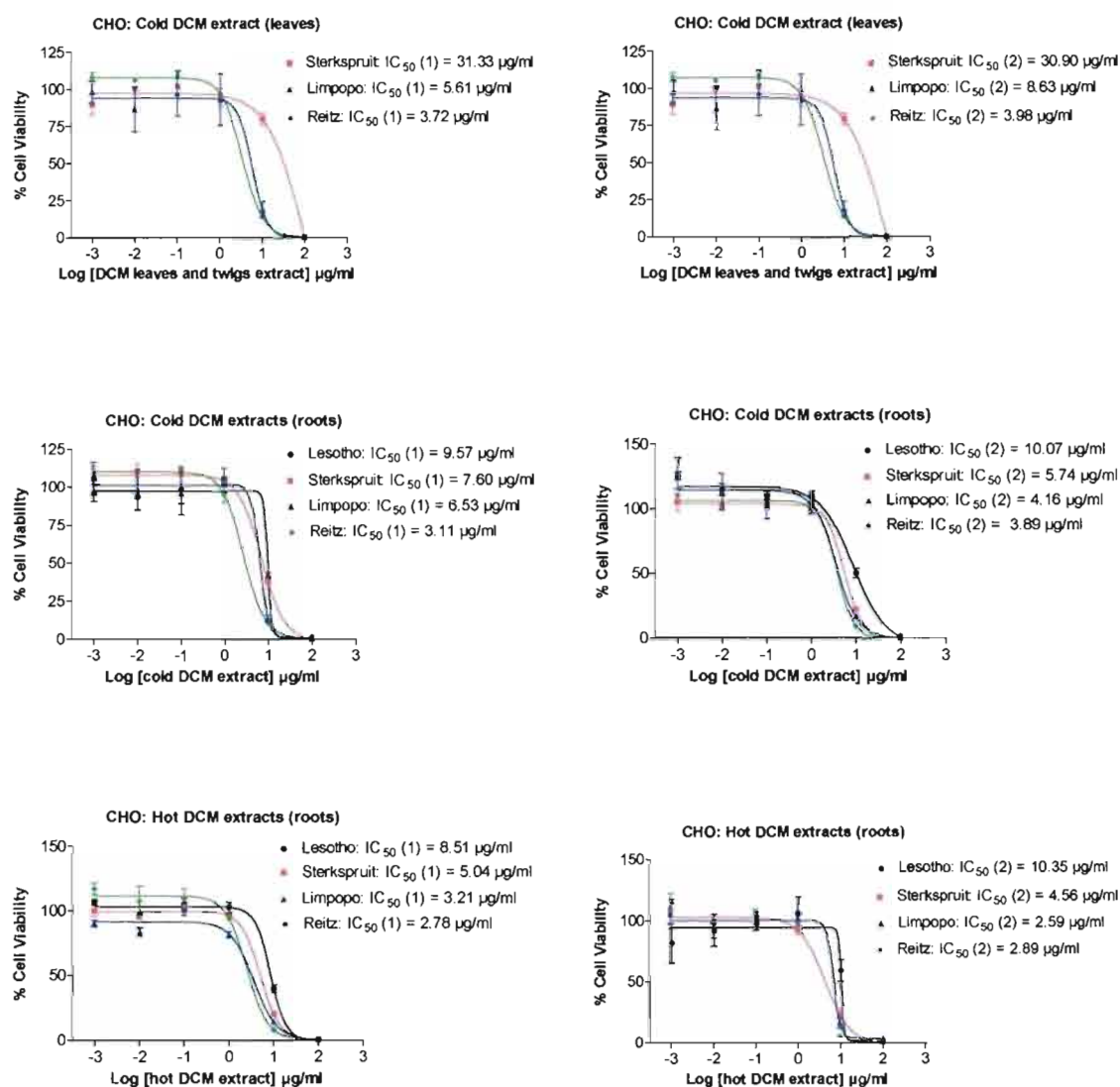
**Figure 5.2a.** The HPLC chromatogram of the cold DCM root extract of *D. anomala* from Sterkspruit.



**Figure 5.2b.** The HPLC chromatogram of the hot DCM root extract of *D. anomala* from Sterkspruit .



**Figure 5.3.** The antiplasmodial dose response curves showing the DCM extracts of the leaves and twigs and the roots of *D. anomala* collected from four locations (Lesotho, Sterkspruit, Reitz and Limpopo).



**Figure 5.4.** The cytotoxicity dose response curves showing the DCM extracts of the leaves and twigs and the roots of *D. anomala* collected from four locations (Lesotho, Sterkspruit, Reitz and Limpopo).

The roots of *D. anomala* from Lesotho were also extracted in other organic solvents (table 5.2). The ethylacetate and chloroform extracts ( $1.56 \pm 0.36 \mu\text{g/ml}$  and  $1.18 \pm 0.08 \mu\text{g/ml}$ ) were active against the D10 strain of *Plasmodium falciparum*. The activity of these extracts was similar to that of the cold DCM

extract ( $1.55 \pm 0.07 \mu\text{g/ml}$ ). The (MeOH) extract showed a marginal activity ( $10.67 \pm 0.32 \mu\text{g/ml}$ ) while the hexane extract ( $39.30 \pm 0.70 \mu\text{g/ml}$ ) and the petroleum spirit extract ( $12.79 \pm 2.44 \mu\text{g/ml}$ ) were inactive against the D10 strain of *P. falciparum*.

**Table 5.2.** The antiplasmodial activity and the cytotoxicity of the organic extracts of *D. anomala* roots from Lesotho on the CQS (D10) strain of *P. falciparum*.

Extract	Polarity Index (PI)	IC <sub>50</sub> mean $\pm$ S.D. ( $\mu\text{g/ml}$ ) D10 strain	IC <sub>50</sub> mean $\pm$ S.D. ( $\mu\text{g/ml}$ ) CHO cells	Selectivity Index (SI)
MeOH	6.6	$10.67 \pm 0.32$	-	-
Hexane	0	$39.30 \pm 0.70$	-	-
Chloroform	4.1	$1.18 \pm 0.08$	$4.84 \pm 0.62$	4.10
Ethylacetate	4.4	$1.56 \pm 0.36$	$16.46 \pm 0.13$	10.55
Petroleum spirit		$12.79 \pm 2.44$	-	-

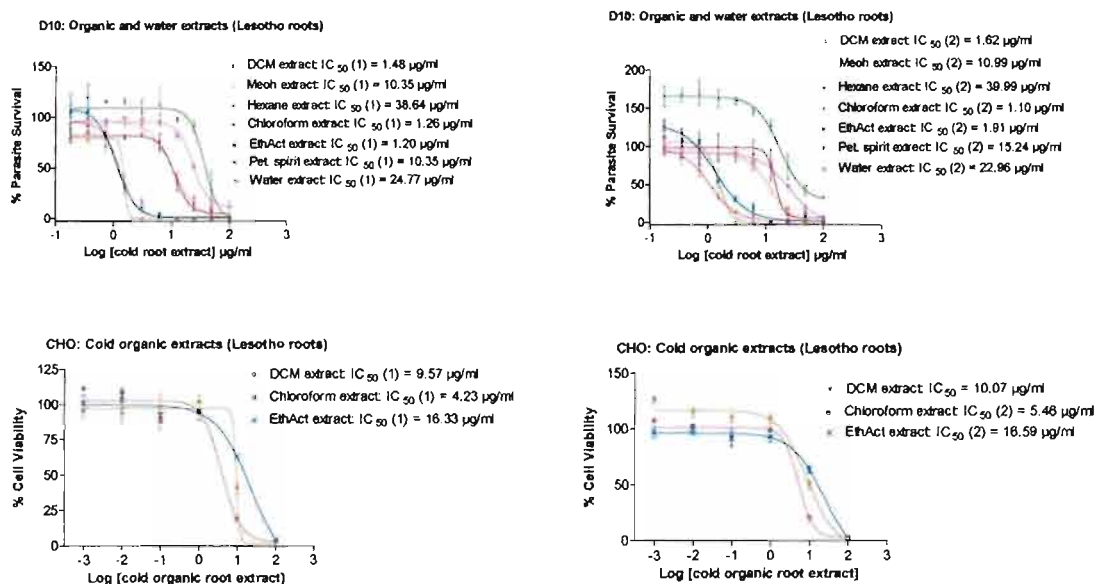
- Not done

The cytotoxicity results showed that the chloroform extract ( $4.84 \pm 0.62 \mu\text{g/ml}$ ) had more cytotoxic effects on the CHO cells than the ethylacetate extract ( $16.46 \pm 0.13 \mu\text{g/ml}$ ). However, the extracts were more potent to the parasites than to the cells as shown by the selectivity indices (chloroform extract = 4.10 and ethylacetate extract = 10.55). The antiplasmodial and cytotoxicity dose response curves of these extracts are shown in figure 5.3.

A comparison of the organic root extracts, using HPLC, indicated that the DCM, ethylacetate and chloroform extracts had similar HPLC profiles hence similar antiplasmodial activity. All the three extracts contained numerous peaks whilst

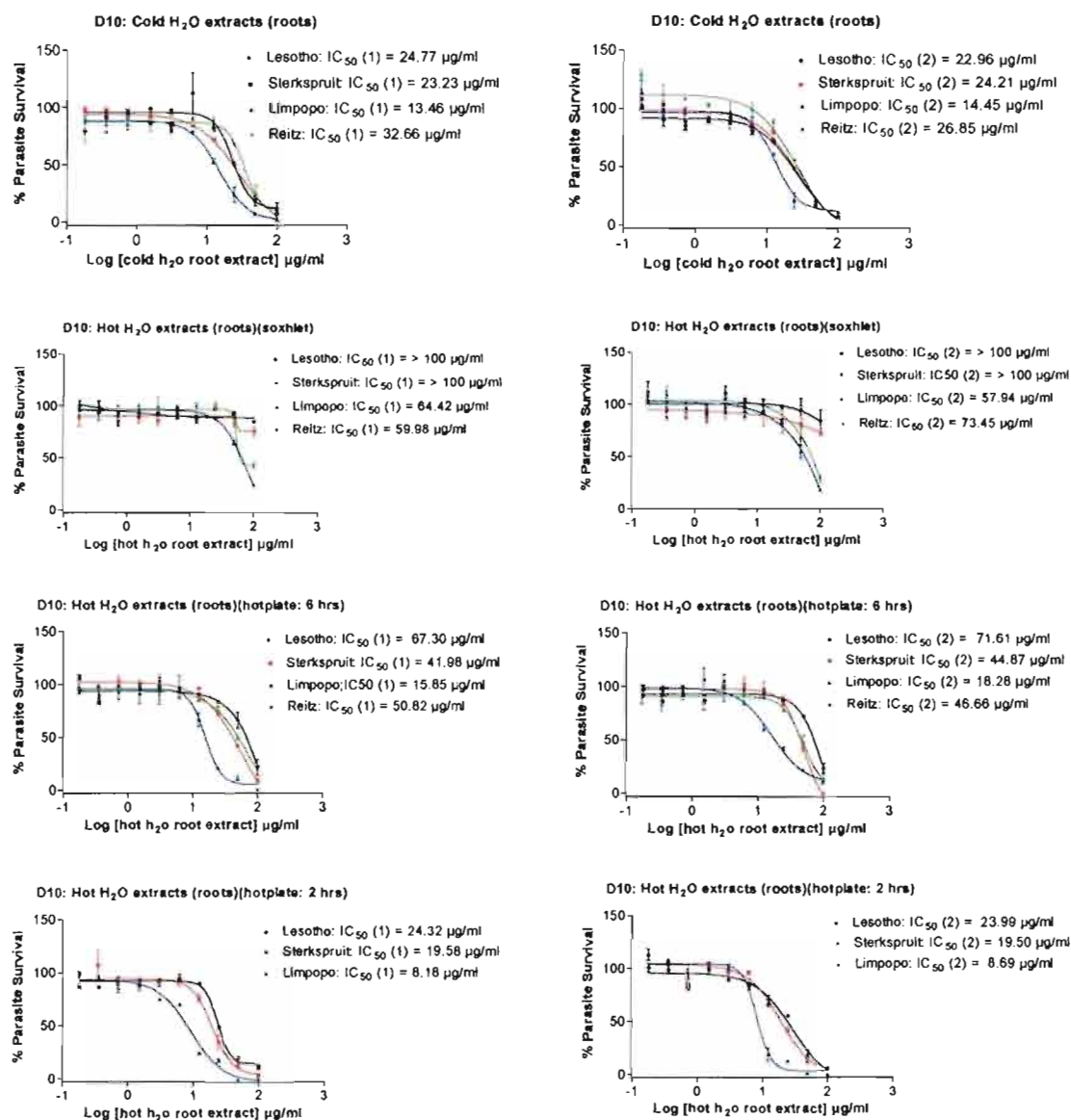


the MeOH, hexane and the petroleum spirit extracts contained fewer peaks (Appendix B). The similarity and the difference in the HPLC profiles of these extracts could be explained by the polarity of the extracts according to the polarity index (PI) of the solvents (table 5.2). Hexane and the petroleum spirit solvents (PI = 0) were more hydrophobic than chloroform, ethylacetate and DCM solvents (PI = 4.4, PI = 4.3 and PI = 3.4, respectively). The MeOH was the only hydrophilic solvent (PI = 6.6).



**Figure 5.5.** The antiparasmodial and cytotoxicity dose response curves of organic and water extracts of the roots of *D. anomala* collected from Lesotho.

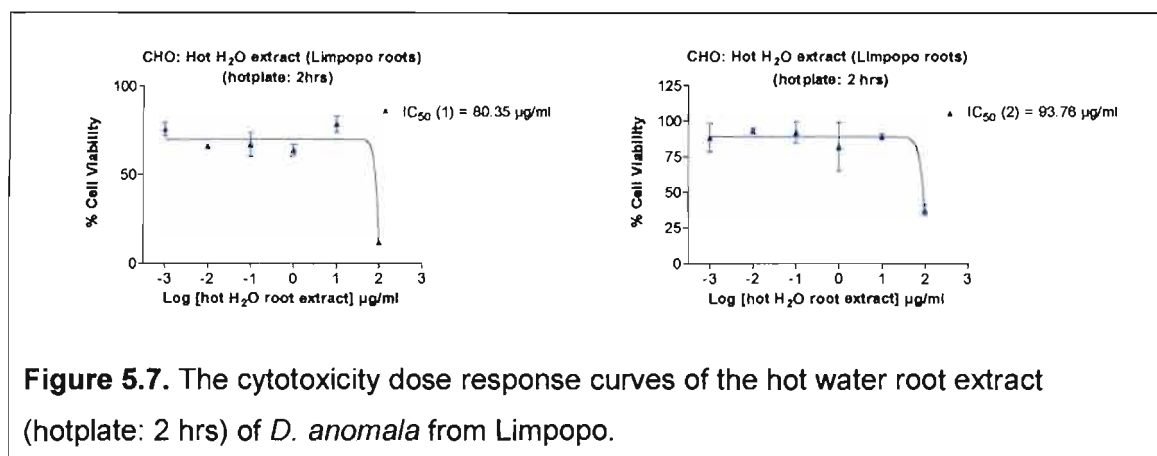
The cold and hot H<sub>2</sub>O root extracts of the roots of *D. anomala* collected from all four locations, did not show antiparasmodial activity against the D10 strain of *P. falciparum* (table 5.4 and figure 5.6) except for the hot H<sub>2</sub>O root extract from Limpopo ( $8.44 \pm 0.25$  µg/ml), prepared on a hotplate for 2 hours.



**Figure 5.6.** The antiparasmodial dose response curves of H<sub>2</sub>O extracts of the roots of *D. anomala* collected from four locations (Lesotho, Sterkspruit, Reitz and Limpopo).

The cytotoxic effect of the hot water root extract from Limpopo (hotplate: 2 hrs,  $87.06 \pm 6.70$  µg/ml) (figure 5.7) was very low on the CHO cells and the selectivity of the antiparasmodial potency was 10 times high towards the parasites. This showed that the antiparasmodial activity of this extract was not associated with the

cytotoxicity on the CHO cells and further supports the treatment of fevers, some of which may be malarial fevers with the roots of *D. anomala*.



**Figure 5.7.** The cytotoxicity dose response curves of the hot water root extract (hotplate: 2 hrs) of *D. anomala* from Limpopo.

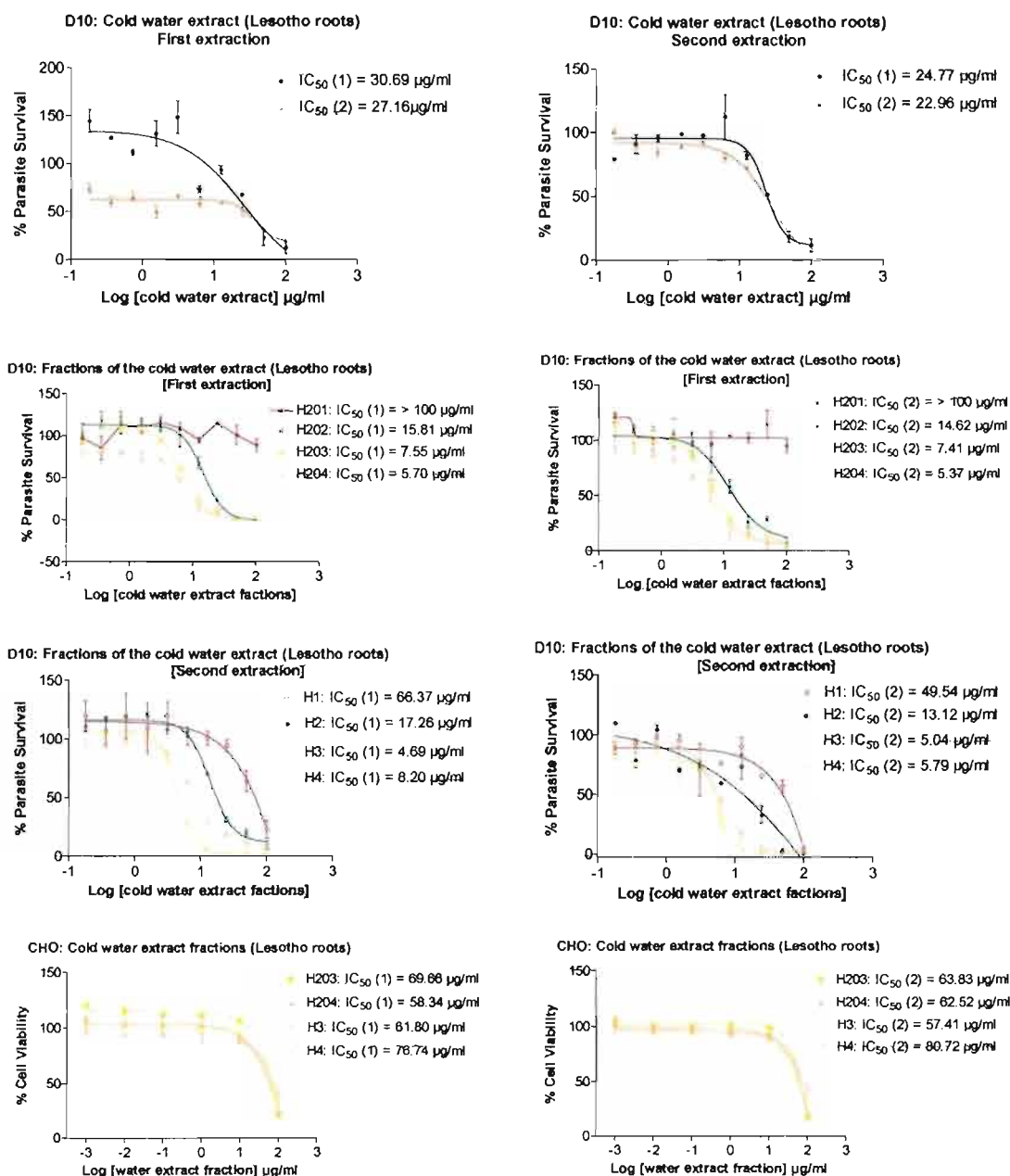
Four fractions (H201 – H204) were obtained from the cold H<sub>2</sub>O root extract from Lesotho. The fractions were obtained according to their colour intensity (details in Chapter 4) and the antiparasmodial activity was found in fractions H203 and H204 ( $7.48 \pm 0.07$  µg/ml and  $5.54 \pm 0.16$  µg/ml) (table 4.3 and figure 5.8). To confirm these results, four other fractions (H1 - H4) were obtained from the second H<sub>2</sub>O extraction of the roots and the antiparasmodial activity was found in fractions H3 and H4 ( $4.85 \pm 0.18$  µg/ml and  $7.0 \pm 1.20$  µg/ml) (table 5.3 and figure 5.8). The antiparasmodial activity of the cold H<sub>2</sub>O root extract ( $23.86 \pm 0.90$  µg/ml), therefore, unfolded when the root extractions were separated into fractions.

The cytotoxic effects of these samples were less on the CHO cells (H203 =  $66.74 \pm 2.92$  µg/ml, H204 =  $60.43 \pm 2.09$  µg/ml, H3 =  $59.60 \pm 2.20$  µg/ml, H4 =  $78.73 \pm 1.99$  µg/ml and the hot H<sub>2</sub>O root extract from Limpopo (hotplate: 2 hrs,  $87.06 \pm 6.70$  µg/ml). The results indicated that the antiparasmodial activity of the samples was not due to the cytotoxicity and the selectivity of the antiparasmodial potency was high by a magnitude of 12 for fractions H204 and H3, 11 for fraction H4 and 9 for fraction H203.

**Table 5.3.** The antiparasmodial activity and cytotoxicity of the fractions of the cold H<sub>2</sub>O root extract of *D. anomala* from Lesotho on the D10 strain of *P. falciparum*.

Extracts and fractions (Roots) (batch II)	IC <sub>50</sub> mean ± S.D. (µg/ml) D10 strain	IC <sub>50</sub> mean ± S.D. (µg/ml) CHO cells	Selectivity Index (SI)
<b>First extraction</b>			
Cold H <sub>2</sub> O extract	28.92 ± 1.76		
H201	> 100	-	-
H202	15.23 ± 0.60	-	-
H203	7.48 ± 0.07	66.74 ± 2.92	8.92
H204	5.54 ± 0.16	60.43 ± 2.09	10.91
<b>Second extraction</b>			
Cold H <sub>2</sub> O extract	23.86 ± 0.90		
H1	57.95 ± 8.42	-	-
H2	15.19 ± 2.07	-	-
H3	4.85 ± 0.18	59.60 ± 2.20	12.26
H4	7.0 ± 1.20	78.73 ± 1.99	11.25

- Not done



**Figure 5.8.** The antiparasmodial and cytotoxicity dose response curves of the cold water root extract and the cold water root extract fractions of *D. anomala* from Lesotho.

The less cytotoxic effects of the H<sub>2</sub>O root extract fractions supports the claim by traditional healers that the H<sub>2</sub>O root extracts of the roots of *D. anomala* are safe although this claim is contradicted by the report that the extracts of *D. anomala* are hepatotoxic [Hedberg and Staugård, 1989]. It is therefore difficult to make any conclusions since it is not known if the extracts that exhibit the hepatotoxicity were organic or H<sub>2</sub>O root extracts or both or the plant was used in a preparation mixture with other plants.

It could be that the extracts screened for hepatotoxicity were organic extracts as it has been shown in this chapter (table 5.6 and figure 5.5) that the active organic extracts had high cytotoxic effects than the active H<sub>2</sub>O fractions. Nevertheless, except for the chloroform extract, all these extracts had similar magnitude of selectivity towards the parasites (table 5.3).

The organic extracts were dissolved in acetonitrile, which had no cytotoxic effects (IC<sub>50</sub> = > 100 µg/ml) on the Chinese Hamster Ovarian (CHO) cells. This therefore rules out the possibility that the cytotoxicity of the organic extracts may be due to the solvent.

**Table 5.4.** Extraction yields of extracts of the leaves and twigs and the roots of *D. anomala* collected from the four locations (Lesotho, Sterkspruit, Limpopo and Reitz).

Extract	Collection Location	Mass extracted (g)	Mass of extract (g)	Extraction yield (%)
<b>Leaves and twigs</b>				
Cold DCM	Sterkspruit	10.69	0.49	4.58%
	Limpopo	12.37	0.72	5.82%
	Reitz	2.21	0.13	5.88%
<b>Roots</b>				
Cold DCM	Lesotho	200.43	3.65	1.82%
	Sterkspruit	100.0	2.71	2.71%
	Limpopo	50.02	2.81	5.62%
	Reitz	50.04	2.38	4.76%
Hot DCM	Lesotho	40.0	0.62	1.55%
	Sterkspruit	40.61	0.53	1.30%
	Limpopo	40.22	0.77	1.91%
	Reitz	20.20	0.30	1.48%
Cold H <sub>2</sub> O	Lesotho	5.15	0.2182	4.24%
	Sterkspruit	10.25	2.05	20.0%
	Limpopo	10.40	1.51	14.52%
	Reitz	10.20	1.60	15.68%
Hot H <sub>2</sub> O (soxhlet)	Lesotho	40.0	12.52	31.3%
	Sterkspruit	40.50	8.57	21.16%
	Limpopo	41.19	10.37	25.18%
	Reitz	17.47	6.31	36.12%
Hot H <sub>2</sub> O (hotplate) 6 hrs	Lesotho	5.0	1.75	35.0%
	Sterkspruit	5.29	1.58	29.87%
	Limpopo	5.37	1.32	24.58%
	Reitz	5.60	1.60	28.57%
Hot H <sub>2</sub> O (hotplate) 2 hrs	Lesotho	5.0	1.70	34.0%
	Sterkspruit	5.24	1.55	29.58%
	Limpopo	5.50	1.35	24.54%

Table 5.4 shows the extraction yields of the DCM and H<sub>2</sub>O extracts from the four locations. All the hot H<sub>2</sub>O root extracts had high yields than the other extracts. The hot H<sub>2</sub>O root extracts from the hotplate had high yields than the hot H<sub>2</sub>O root

extract from the soxhlet and the cold H<sub>2</sub>O root extract. In the organic extracts (table 5.5 below), the MeOH extract had the highest yield followed by the hot DCM extract (table 5.1 and 5.2). This means that there are more compounds extracted in H<sub>2</sub>O, in particular, hot H<sub>2</sub>O than in organic extracts. In addition, more compounds were extracted with MeOH than with other organic solvents used for the extraction. Most compounds extracted from the roots of *D. anomala* are therefore polar compounds since the H<sub>2</sub>O root extracts, in particular hot H<sub>2</sub>O root extracts and the MeOH extract had the highest yields. This is supported by the fact that H<sub>2</sub>O and MeOH are more polar than the other organic solvents used.



**Table 5.5.** Extraction yields of organic extracts and fractions of the cold water root extract of the roots of *D. anomala* from Lesotho.

Extracts	Mass extracted (g)	Mass of extract (g)	Extraction yield (%)
Methanol	5.15	0.84	16.31%
Hexane	5.01	0.03	0.60%
Chloroform	5.02	0.11	2.19%
Ethylacetate	5.02	0.16	3.19%
Petroleum spirit	5.18	0.05	0.96%
<b>Water root extract fractions</b>			
<b>First extraction</b>	5.16		
H201		0.821	15.91%
H202		0.039	0.76%
H203		0.028	0.54%
H204		0.016	0.31%
<b>Second extraction</b>	5.15		
H1		0.771	14.9%
H2		0.034	0.65%
H3		0.029	0.56%
H4		0.016	0.31%

## 5.4. CONCLUSION

- The antiplasmodial activity of the DCM leaf and twig extracts of *D. anomala* has confirmed their use for the treatment of malaria and fever [Neuwinger, 2000].
- The use of *D. anomala* for the treatment of fevers, some of which could be malarial fevers, was also confirmed by the antiplasmodial activity of the organic extracts of the roots (cold and hot DCM extracts, chloroform, petroleum and ethylacetate extracts).
- Hot and cold H<sub>2</sub>O root extracts did not show antiplasmodial activity except for the hot H<sub>2</sub>O root extract from Limpopo. The cold H<sub>2</sub>O root extract from Lesotho have been shown to possess antiplasmodial activity only when it was separated into fractions. The activity was found in fractions (H203, H204, H3 and H4) which also showed less cytotoxicity.
- The roots of *D. anomala* have been shown to contain more polar compounds as observed from the high yields of the H<sub>2</sub>O and the MeOH extracts.
- Further work has to be done on the organic extracts and the H<sub>2</sub>O root extract fractions from *D. anomala*. Compounds have to be isolated and screened for antiplasmodial activity to determine which compounds are responsible for the activity and also determine their cytotoxicity, *in vivo* antimalarial activity and *in vivo* toxicity.

## **CHAPTER 6**

### **BIOACTIVITY-GUIDED FRACTIONATION OF THE CRUDE DICHLOROMETHANE ROOT EXTRACT OF**

***Dicoma anomala***

## 6.1. INTRODUCTION

The hot and cold DCM extracts of the roots of *D. anomala* from Lesotho had similar antiplasmodial activity but only the cold DCM extract was fractionated.

The cold DCM root extracts from batch I and II were subjected to solid phase extraction to obtain 10% and 5% fractions. In addition, 1% fractions were also obtained from the cold DCM root extract from batch I. The cold DCM root extract from Sterkspruit had similar antiplasmodial activity and similar HPLC profile to the one from Lesotho and was therefore, also subjected to SPE to obtain three fractions (fraction 30, 40 and 50). These fractions were the most active fractions in the bio-activity guided fractionation of the cold DCM root extract (batch I and II) from Lesotho.

## 6.2. METHODS

The cold DCM extract of the roots was subjected to bio-activity guided fractionation using SPE. The fractionation was carried out using the Isolute C18 (EC) cartridge of mass absorbent 10 g and 70 ml capacity, which was conditioned sequentially with 50 ml methanol and 50 ml Millipore water to facilitate binding of compounds. A 50 ml aliquot of the 4 mg/ml crude DCM extract solution was then applied onto the column and eluted under weak vacuum at a flow rate of 17 ml/min.

A step gradient of increasing 10% mobile phases of acetonitrile and water (MeCN:H<sub>2</sub>O) was used to elute bound compounds to afford 13 fractions (percolate, water wash, fractions 10 – 100 and acetone wash).

To achieve fractions with single peaks, further fractionation was carried out with a step gradient of 5% and 1% increment mobile phases of MeCN:H<sub>2</sub>O in the range 35% - 55% and 31% - 35% respectively. The details of the methods are in Chapter 4.

### 6.3. RESULTS AND DISCUSSION

**Table 6.1.** Extraction yields of 10% fractions from batch I and batch II of the cold DCM root extract of *D. anomala* from Lesotho.

10% SPE Fractions	Batch I Cold DCM extract (220 mg )		Batch II Cold DCM extract (280 mg)	
	Mass obtained (mg)	% Yield	Mass obtained (mg)	% Yield
Percolate	43.80	19.91	58.88	21.03
Water wash	2.90	1.32	4.68	1.67
10	4.35	1.98	3.03	1.08
20	23.13	10.51	12.85	4.59
30	50.58	22.99	50.62	18.08
40	21.07	9.58	23.13	8.26
50	3.55	1.61	7.11	2.54
60	2.24	1.02	2.59	0.92
70	2.12	0.96	7.38	2.64
80	1.12	0.51	4.13	1.48
90	1.07	0.49	1.08	0.38
100	1.10	0.50	2.47	0.88
Acetone wash	6.20	2.82	4.45	1.59

The extraction yields obtained for the SPE fractions of the cold DCM root extract of *D. anomala* (batch I and II) from Lesotho were lower than 24% (table 6.1 and 6.2). Although more material of the cold DCM root extract was loaded (400 mg and 662 mg, respectively) to obtain 5% and 1% fractions, as compared to 280

mg for the 10% fractions, fractions with high yields (4.59% - 22.99%) were found in the 10% SPE fractions (percolate, fractions 20, 30 and 40). There were however, variations in the % yields of fractions 10, 20, 70, 80 and acetone wash of batch I and II. This was also observed with the % yields of fraction 50 from the 5% fractions. These could be explained as operator inconsistencies. As the cold DCM root extract was further purified, lower yields of the fractions were obtained. Purifying crude extracts has been a major challenge because of the low yields of fractions and compounds.

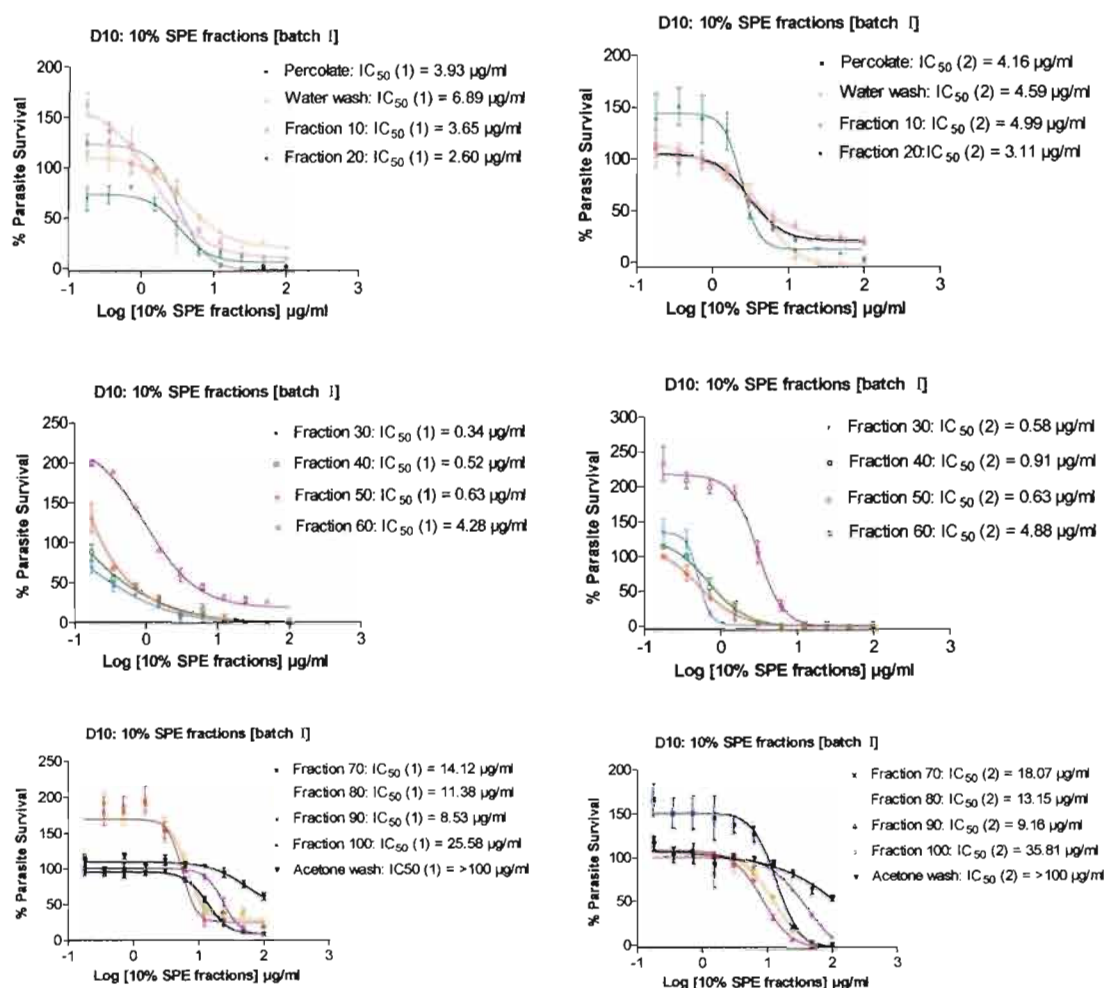
**Table 6.2.** Extraction yields of 1% and 5% fractions from batch I and batch II of cold DCM root extract of *D. anomala* from Lesotho.

Cold DCM extract (Batch I) (310 mg)			Cold DCM extract (Batch II) (400 mg)	
5% SPE Fractions	Mass obtained (mg)	% Yield	Mass obtained (mg)	% Yield
35	20.45	6.60	18.05	4.51
40	13.08	4.22	16.09	4.02
45	3.46	1.12	4.96	1.24
50	2.61	0.84	1.94	0.48
55	1.39	0.45	1.49	0.37
Cold DCM extract (Batch I) (662 mg)				
1% SPE Fractions	Mass obtained (mg)	% Yield		
31	70.3	10.62		
32	69.3	10.47		
33	31.8	4.80		
34	43.9	6.63		
35	50.1	7.57		

**Table 6.3.** The *in vitro* antiparasmodial activity of 10% and 5% SPE fractions from batch I and II of the cold DCM root extract of *D. anomala* from Lesotho on CQS (D10) strain of *P. falciparum* and the *in vitro* cytotoxicity of fractions 40 and 50 on CHO cells.

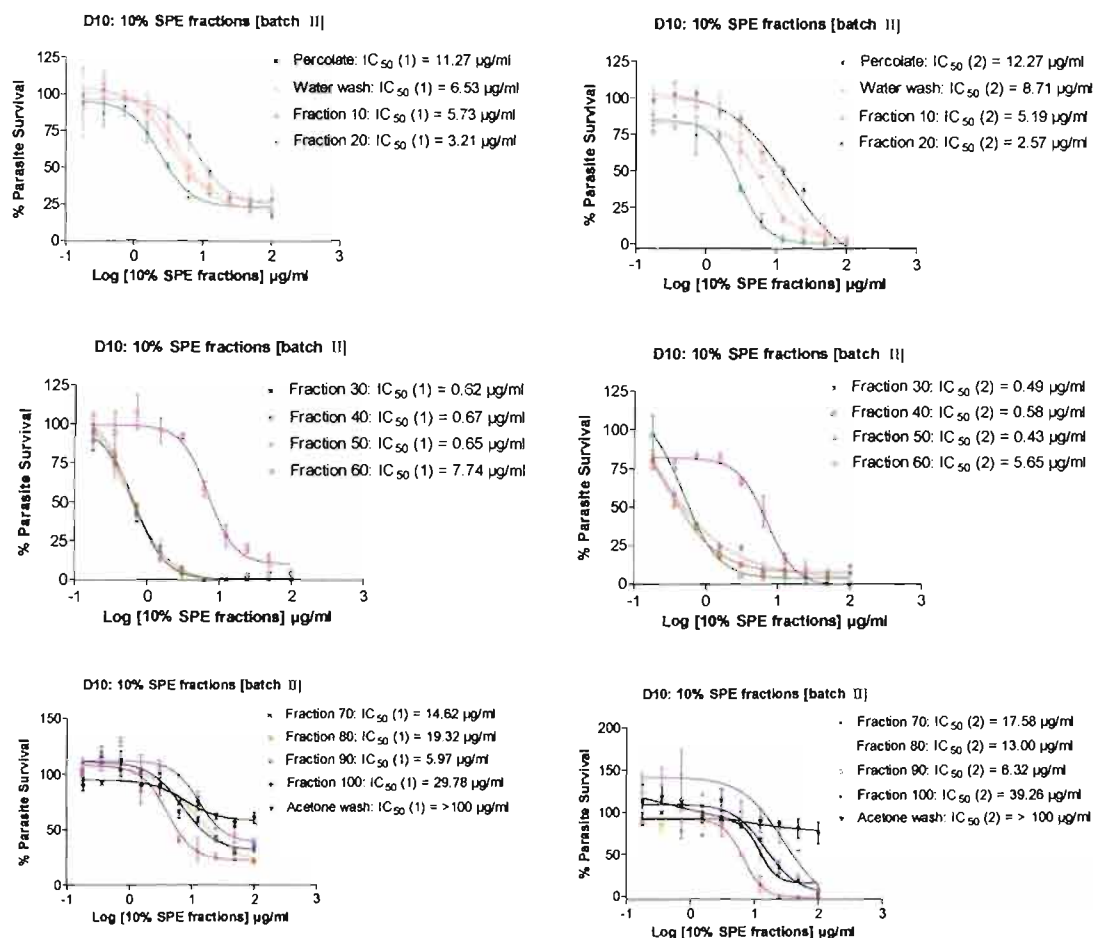
	<b>Batch I Cold DCM extract</b>	<b>Batch II Cold DCM extract</b>	<b>Batch II Cold DCM extract</b>
<b>10% SPE Fractions</b>	<b>IC<sub>50</sub> ± SD (µg/ml) D10 strain</b>	<b>IC<sub>50</sub> ± SD (µg/ml) D10 strain</b>	<b>IC<sub>50</sub> ± SD (µg/ml) CHO cells</b>
Percolate	4.04 ± 0.12	11.77 ± 0.50	
Water wash	5.74 ± 1.15	7.62 ± 1.09	
10	4.32 ± 0.67	5.46 ± 0.27	
20	2.90 ± 0.30	2.89 ± 0.32	
30	0.46 ± 0.12	0.56 ± 0.065	
40	0.72 ± 0.20	0.63 ± 0.045	3.76 ± 0.27 (SI = 5.97)
50	0.63 ± 0.1	0.54 ± 0.11	3.50 ± 0.13 (SI = 6.48)
60	4.58 ± 0.30	6.70 ± 1.05	
70	16.04 ± 1.92	16.10 ± 1.48	
80	12.27 ± 0.89	16.16 ± 3.16	
90	8.85 ± 0.32	6.15 ± 0.18	
100	30.70 ± 5.12	34.52 ± 4.74	
Acetone wash	> 100	> 100	
<b>5% SPE Fractions</b>			
35	0.56 ± 0.09	0.23 ± 0.025	
40	0.50 ± 0.06	0.67 ± 0.065	
45	0.38 ± 0.02	0.29 ± 0.01	
50	0.81 ± 0.04	0.40 ± 0.02	
55	1.09 ± 0.005	0.56 ± 0.005	

The water wash, fractions 10 - 60 and 90 from batch I and batch II of the cold DCM root extract from Lesotho showed antiplasmodial activity against the CQS (D10) strain of *P. falciparum*. The most active fractions were fractions 30, 40 and 50. Fractions 70, 80, 100 and the acetone wash from both batches did not show significant antiplasmodial activity (table 6.3 and figures 6.1a and 6.1b).



**Figure 6.1a.** The dose response curves showing the antiplasmodial activity of 10% SPE fractions from the cold DCM root extract (batch I) of *D. anomala* from Lesotho.



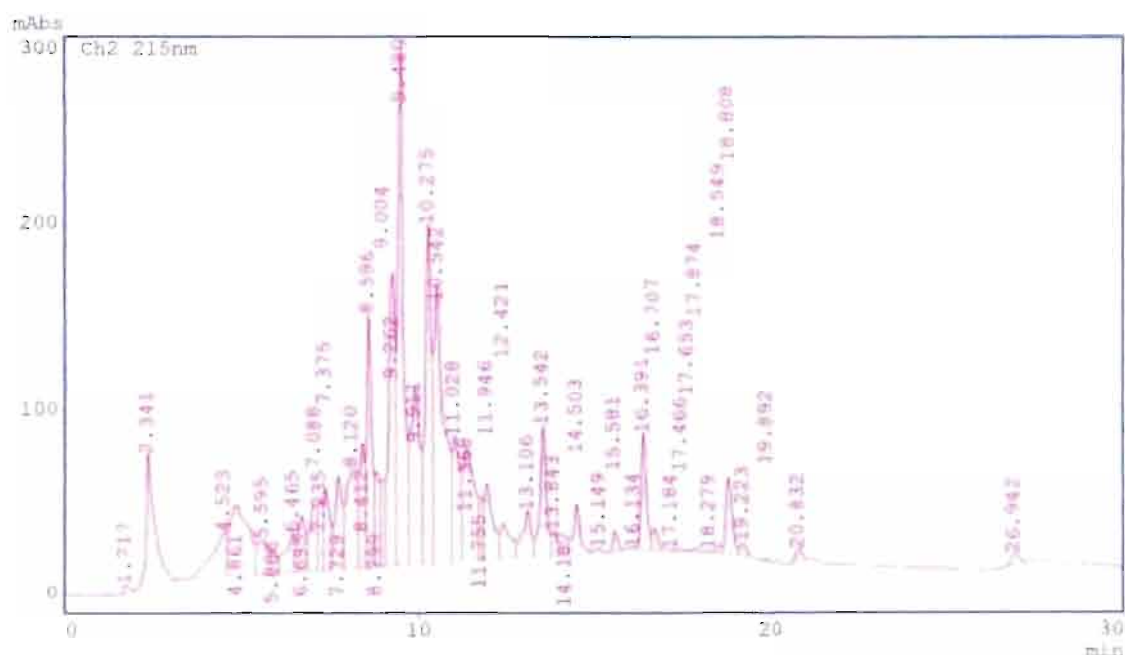


**Figure 6.1b.** The dose response curves showing the antiplasmodial activity of 10% SPE fractions from the cold DCM root extract (batch II) of *D. anomala* from Lesotho.

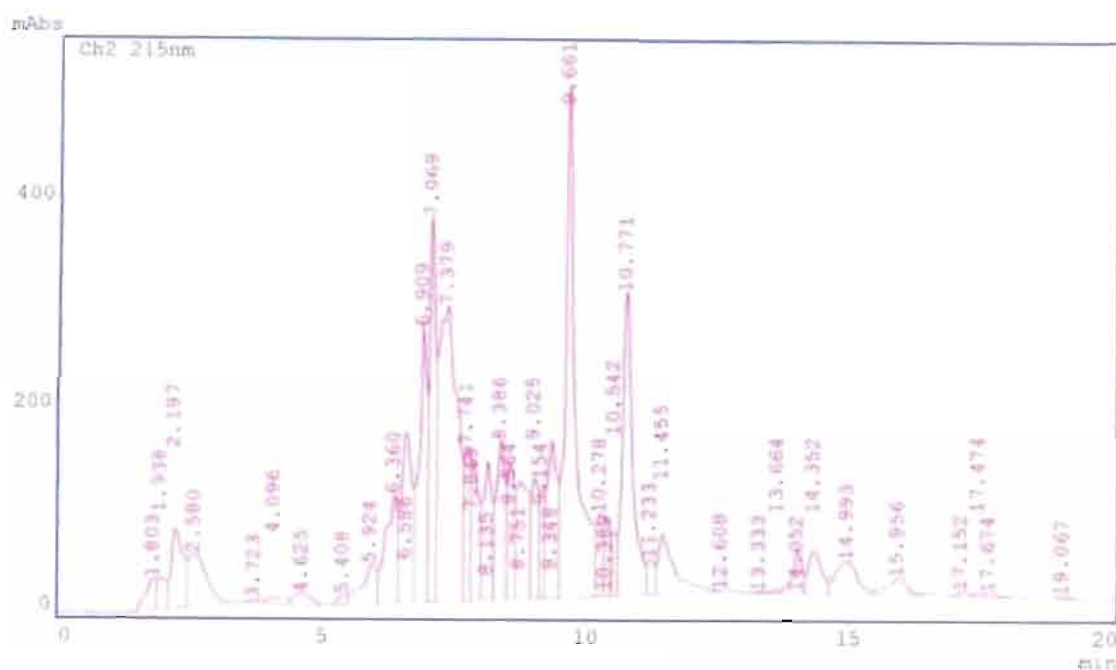
Compounds that did not bind to the SPE column were washed off with water, which means that the water wash ( $5.74 \pm 1.15 \mu\text{g/ml} \leq IC_{50} \leq 7.62 \pm 1.09 \mu\text{g/ml}$ ) contained polar compounds with antiplasmodial activity. The antiplasmodial activity of the water extract fractions (H3, H4, H203 and H204) which was between  $4.85 \pm 0.5 \mu\text{g/ml}$  and  $7.48 \pm 0.07 \mu\text{g/ml}$  (table 5.3, Chapter 5), has confirmed that the roots of *D. anomala* contained polar compounds which are active against *P. falciparum*.

Fractions 30, 40 and 50 were more active ( $0.46 \pm 0.12 \mu\text{g/ml} \leq \text{IC}_{50} \leq 0.72 \pm 0.20 \mu\text{g/ml}$ ) than the cold DCM extract from batch I ( $2.48 \pm 0.005 \mu\text{g/ml}$ ) and batch II ( $1.55 \pm 0.07 \mu\text{g/ml}$ ) of the roots of *D. anomala* (table 4.1, Chapter 4). This showed that the activity of the cold DCM root extracts was not lost due to fractionation and that it was more likely to be due to the activity of individual compounds rather than synergism between the compounds.

The percolate from batch I of the cold DCM root extract exhibited antiplasmodial activity ( $4.05 \pm 0.12 \mu\text{g/ml}$ ) whilst that from batch II did not show significant antiplasmodial activity ( $11.77 \pm 0.50 \mu\text{g/ml}$ ). The HPLC profiles of these percolates were different and showed that the active percolate contained more peaks than the inactive one (figures 6.2a and 6.2b) and the HPLC profiles were different.



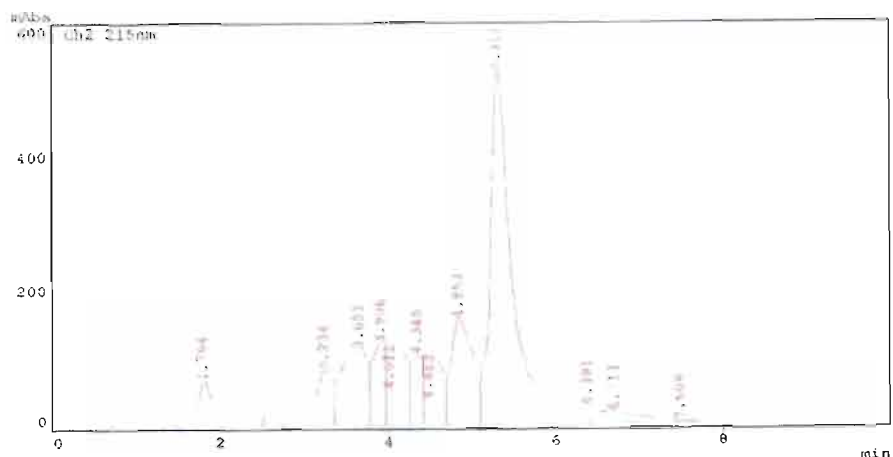
**Figure 6.2a.** The HPLC chromatogram of the percolate from the cold DCM root extract (batch I) of *D. anomala* from Lesotho. The analytical HPLC run was on a Phenomenex column (150 x 4.6 mm, 5  $\mu\text{m}$ ) at a gradient of 20% - 100% MeCN in  $\text{H}_2\text{O}$  and a flow rate of 1ml/min. The concentration was 1 mg/ml and the injection volume was 100  $\mu\text{l}$ .



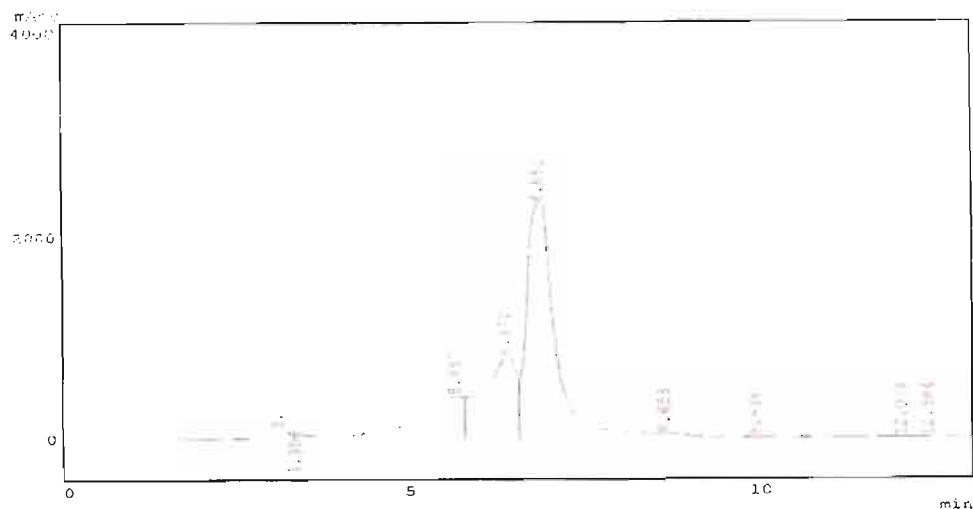
**Figure 6.2b.** The HPLC chromatogram of the percolate from the cold DCM root extract (batch II) of *D. anomala* from Lesotho. The analytical HPLC run was on a Phenomenex column (150 x 4.6 mm, 5  $\mu$ m) at a gradient of 20% - 100% MeCN in H<sub>2</sub>O and a flow rate of 1ml/min. The concentration was 1 mg/ml and the injection volume was 100  $\mu$ l.

The SPE fractionation of the cold DCM root extract (batch II) into 10% fractions was performed to determine if it was reproducible when compared to fractions from batch I of the cold DCM root extract. There was no significant difference in the antiplasmodial activity of 10% fractions from both batches, except for the percolate. Fractions 30, 40 and 50 were still the most active fractions. Although fractions 40 ( $3.76 \pm 0.27$   $\mu$ g/ml, SI = 5.97) and 50 ( $3.50 \pm 0.13$   $\mu$ g/ml, SI = 6.48) were cytotoxic, the effect was more towards the parasites than to the CHO cells.

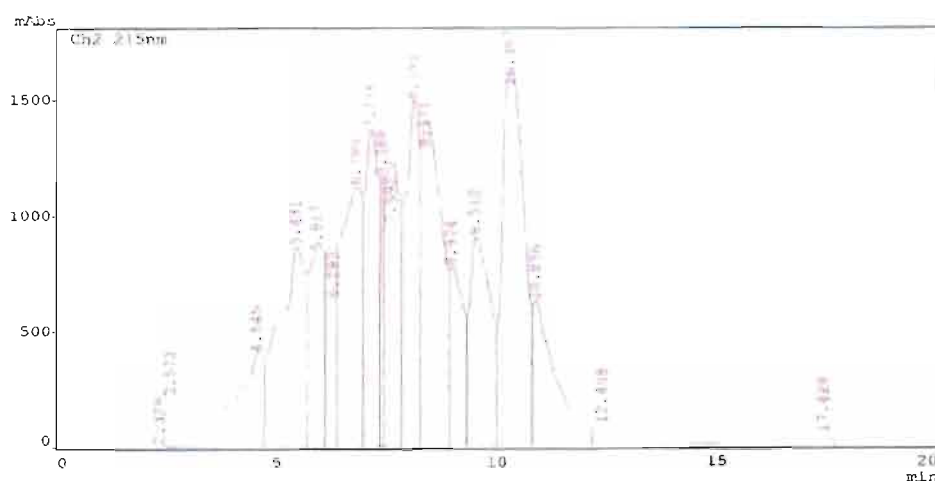
The SPE fractionation of the cold DCM root extract of *D. anomala* (batch I and batch II) into 10% fractions was reproducible and the antiplasmodial activity of the cold DCM root extracts was not lost due to fractionation. The reproducibility of SPE fractionation of the cold DCM root extracts (batch I and batch II) is shown in figures 6.3a – f by the HPLC chromatograms of the most active 10% SPE fractions (fractions 30, 40 and 50).



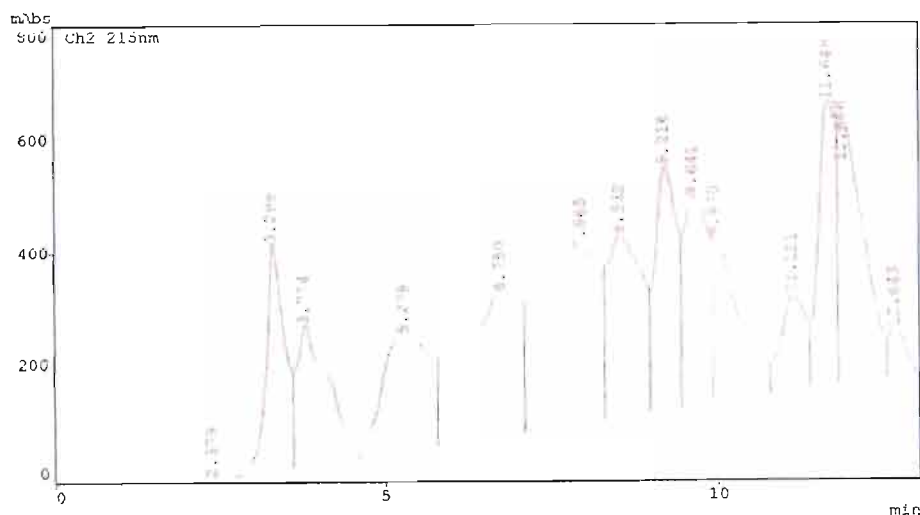
**Figure 6.3a.** The HPLC chromatogram of fraction 30 (1 mg/ml) from the cold DCM root extract (batch I) of *D. anomala* from Lesotho. The fraction was run at a gradient of 50% - 70% MeCN: H<sub>2</sub>O on a C18 reverse-phase analytical Haisil column (250 x 4.6 mm, 5  $\mu$ m), at a flow rate of 1 ml/min and were monitored at 215 nm. The injection volume was 150  $\mu$ l.



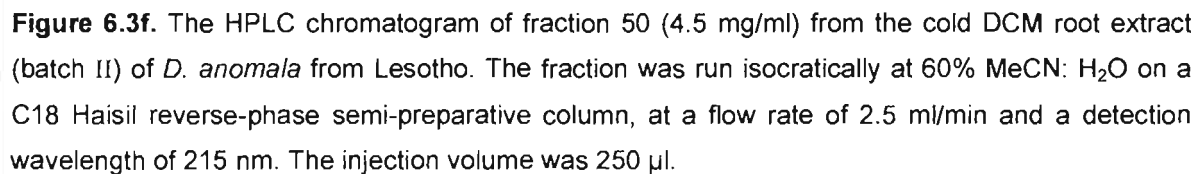
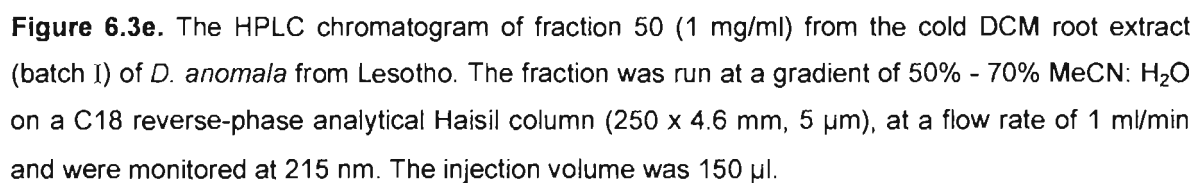
**Figure 6.3b.** The HPLC chromatogram of fraction 30 (4.5 mg/ml) from the cold DCM root extract (batch II) of *D. anomala* from Lesotho. The fraction was run isocratically at 60% MeCN: H<sub>2</sub>O on a C18 Haisil reverse-phase semi-preparative column, at a flow rate of 2.5 ml/min and a detection wavelength of 215 nm. The injection volume was 250  $\mu$ l.



**Figure 6.3c.** The HPLC chromatogram of fraction 40 (1 mg/ml) from the cold DCM root extract (batch I) of *D. anomala* from Lesotho. The fraction was run at a gradient of 50% - 70% MeCN: H<sub>2</sub>O on a C18 reverse-phase analytical Haisil column (250 x 4.6 mm, 5  $\mu$ m), at a flow rate of 1 ml/min and were monitored at 215 nm. The injection volume was 150  $\mu$ l.

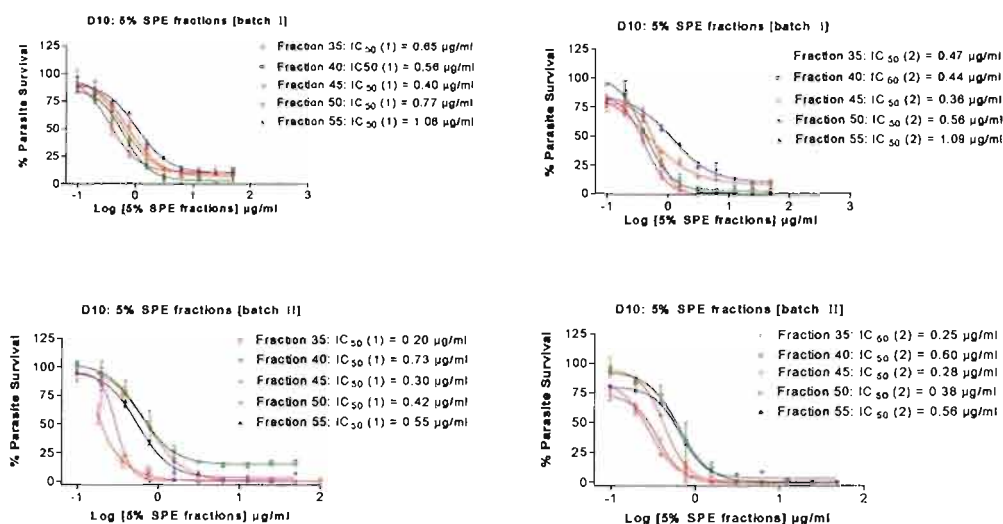


**Figure 6.3d.** The HPLC chromatogram of fraction 40 (4.5 mg/ml) from the cold DCM root extract (batch II) of *D. anomala* from Lesotho. The fraction was run isocratically at 60% MeCN: H<sub>2</sub>O on a C18 Haisil reverse-phase semi-preparative column, at a flow rate of 2.5 ml/min and a detection wavelength of 215 nm. The injection volume was 250  $\mu$ l.



The HPLC profiles of the most active 10% SPE fractions (fractions 30, 40 and 50) showed that these fractions were not pure compounds. The cold DCM root extract was further purified into 5% fractions in order to obtain pure compounds. Only fractions 40 and 50 were fractionated further since fraction 30 was part of an ongoing research in the Division of Pharmacology, University of Cape Town [Matsabisa, PhD thesis]. SPE was then performed using 5% increments of MeCN:H<sub>2</sub>O in the range 35% - 55% to expand fractions 40 and 50.

As done with the 10% fractions, the 5% fractions were generated from both batches (batch I and II) of the cold DCM root extract of *D. anomala*. The fractions appeared fluffy and cream in colour and their *in vitro* antiplasmodial activity of the 5% fractions is shown in table 5.3 and figure 6.4. All fractions were active ( $0.23 \pm 0.025 \mu\text{g/ml} \leq \text{IC}_{50} \leq 1.09 \pm 0.005 \mu\text{g/ml}$ ) against the D10 strain of *P. falciparum*. The purity of the 5% SPE fractions was checked on the HPLC and all fractions contained a number of peaks and were therefore not pure compounds. Fractions 35 – 50 from both batch I and II had similar HPLC profiles while the HPLC profiles of fraction 55 (batch I and batch II) were different. The HPLC profiles of fractions 35 – 55 from batch I are shown in Appendix C.



**Figure 6.4.** The dose response curves showing the antiplasmodial activity of 5% SPE fractions from the cold DCM root extract (batch I and II) of *D. anomala* from Lesotho.

The similarity or difference in the HPLC profiles of 10% and 5% fractions from both batches could explain why some fractions were more active than others and other fractions had similar antiplasmodial activities.

The similar HPLC profiles of fractions 35 – 50 and the antiplasmodial activity on the D10 strain has shown that the SPE fractionation of the cold DCM root extract of *D. anomala* into 5% fractions was reproducible and that the activity of the cold DCM root extract, fractions 40 and 50 was retained. This further indicates that the compounds in the cold DCM root extract may not be acting in synergy for the antiplasmodial activity.

To further achieve purification of the compounds, 1% fractions generated only from the cold DCM root extract of batch I. The SPE fractionation was carried out using 1% increments of MeCN:H<sub>2</sub>O mobile phases in the range 31% - 35%. Fraction 35 was the first fraction generated in the 5% fractions and its peaks were well separated, therefore the 1% SPE fractions generated were fractions 31 – 35.



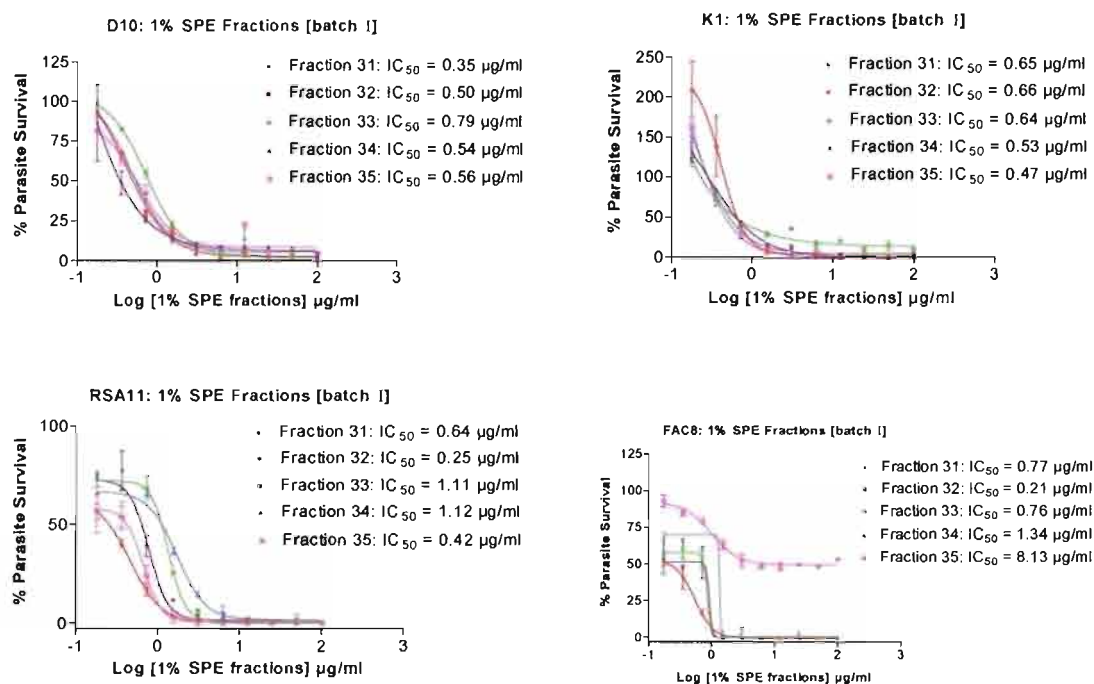
In order to optimise separation of 1% fractions and generate pure samples, 300 ml volumes of 31% - 35% MeCN:H<sub>2</sub>O mobile phases were used as opposed to 200 ml volumes used for the elution of 10% and 5% SPE fractions. The higher volumes of elution were used to wash off any compounds that may be carried over to the next fraction, thereby obtaining pure compounds or fractions with very few peaks. The 1% SPE fractions were fluffy and white in colour as compared to cream for the 5% SPE fractions but the fractionation and the high volume of elution (300 ml) did not result in pure compounds. This was revealed by their HPLC profiles (Appendix C), which showed a lot of peaks compared to those of the 5% fractions (fraction 35 and 40). This means that more components were eluted with the 1% mobile phases of MeCN:H<sub>2</sub>O, indicating that the components in the cold DCM root extract were more separated with 1% mobile phases of MeCN:H<sub>2</sub>O. These components could have been co-eluting when fractionating with the 10% mobile phases of MeCN:H<sub>2</sub>O.

To optimize the separation of the compounds in fraction 31, a TLC method was tried. The compounds in the fraction were eluted with benzene: acetone (50:50), benzene: ethylacetate (30:70), benzene: acetic acid (60:40), chloroform: methanol (50:50) and ethylacetate: acetate acid (60:40). More bands were observed with the benzene: acetic acid (60:40) mobile phase. The R<sub>f</sub> values of the bands are shown in table 6.4. About 7 bands were observed at 254 nm and 5 at 366 nm, although most were faint bands. The concentrated bands (bands 3 and 5 at 254 nm and bands 3 and 6 at 365 nm) were scraped off and their purity checked on the HPLC. The HPLC profile of each band showed that there were about 6 peaks present (Appendix D). The TLC method did not therefore, result in pure compounds.

**Table 6.4.** The Rf values of bands from fraction 31 ran in benzene: acetic acid mobile phase on an aluminium coated TLC plate. The bands highlighted in blue were the most concentrated ones.

Distance travelled by the mobile phase = 18.6 cm		
Band number at 254 nm	Distance travelled (cm)	Rf values
1	1.5	0.081
2	2.3	0.12
3	4.1	0.22
4	6.9	0.37
5	12.1	0.65
Band number at 365 nm		
1	4.3	0.23
2	6.2	0.33
3	7.0	0.38
4	9.8	0.53
5	11.5	0.62
6	12.1	0.65
7	12.7	0.68

Fractions 31 – 35 were tested for *in vitro* antiplasmodial activity against the CQS (D10), CQR (K1, RSA11 and FAC8) strains of *P. falciparum* and all fractions showed good activity against all four strains of the malarial parasites (figure 6.5). As with the 10% and 5% SPE fractions, the fractionation of the cold DCM root extract into 1% fractions did not result in the loss of antiplasmodial activity against the D10 strain.



**Figure 6.5.** The dose response curves showing the antiplasmodial activity of 1% SPE fractions from the cold DCM root extract (batch I) of *D. anomala* from Lesotho.

Although all fractions were active against the D10, K1, RSA11 and FAC8 strains. Fraction 31, 32 and 35 were the most active fractions against the respective strains. Fraction 31 was most active against D10 strain, fraction 35 against K1 strain and fraction 32 against RSA11 and FAC8 strains. Fraction 35 showed the lowest activity against FAC8 strain. The difference in the antiplasmodial activity of the fractions acting against the same strain could be attributed to differences in the compounds or their active sites.

Some fractions showed similar antiplasmodial activity against the same strain of *P. falciparum* (fractions 32, 34 and 35 against D10 strain; fractions 31 – 33 against K1 strain, fractions 33 and 34 against RSA11 strain and fractions 31 and 33 against FAC8 strain. These findings suggest that the fractions might have the

similar active sites. There were also fractions which showed similar antiparasmodial activity against the CQS and on CQR strains (fraction 33 against D10 and FAC 8 and fraction 34 against D10 and K1 strains), indicating that these fractions might have a different mechanism of action to that of chloroquine. Others showed similar antiparasmodial activity against two CQR strains (fractions 32 and 34 against RSA11 and FAC8 strains and fractions 31 and 35 against K1 and RSA11 strains), suggesting that the fractions might not be sharing the mechanism of resistance of chloroquine. The results of fractions 31 – 35 obtained against both CQS and CQR strains have shown that these fractions are a potential source of compounds that could be used for the treatment of both sensitive and resistant strains of *P. falciparum*.

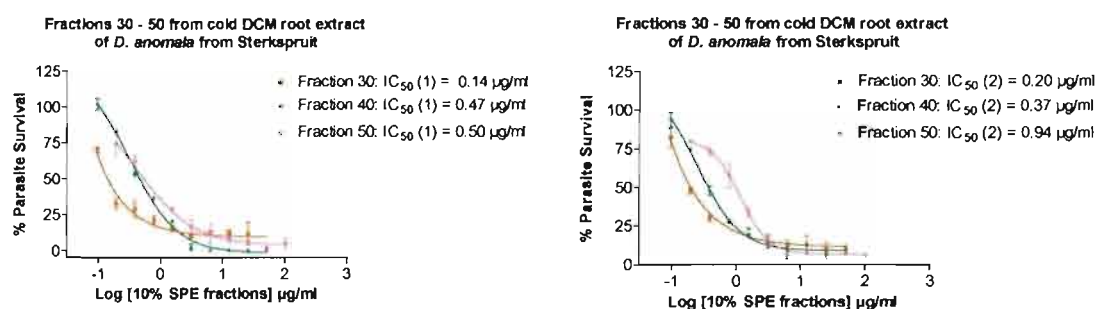
**Table 6.5.** The % yields and the antiparasmodial activity of 10% SPE fractions from the cold DCM root extract of *D. anomala* from Sterkspruit on the D10 strain of *P. falciparum*.

10% SPE Fractions	Mass loaded = 1.02 g		IC <sub>50</sub> ± SD IC <sub>50</sub> (µg/ml)
	Mass obtained (mg)	% Yields	
30	200.41	19.65%	0.17 ± 0.03
40	52.19	5.12%	0.42 ± 0.05
50	13.55	1.33%	0.72 ± 0.22

The cold DCM root extract of *D. anomala* (cold extraction) from Sterkspruit had a similar HPLC profile to the one from Lesotho (figure 5.1a, Chapter 5). It was therefore fractionated into fractions 30, 40 and 50 to see if these will have similar antiparasmodial activity and HPLC profiles to those obtained from the cold DCM root extract of *D. anomala* from Lesotho. These three fractions (30, 40 and 50)

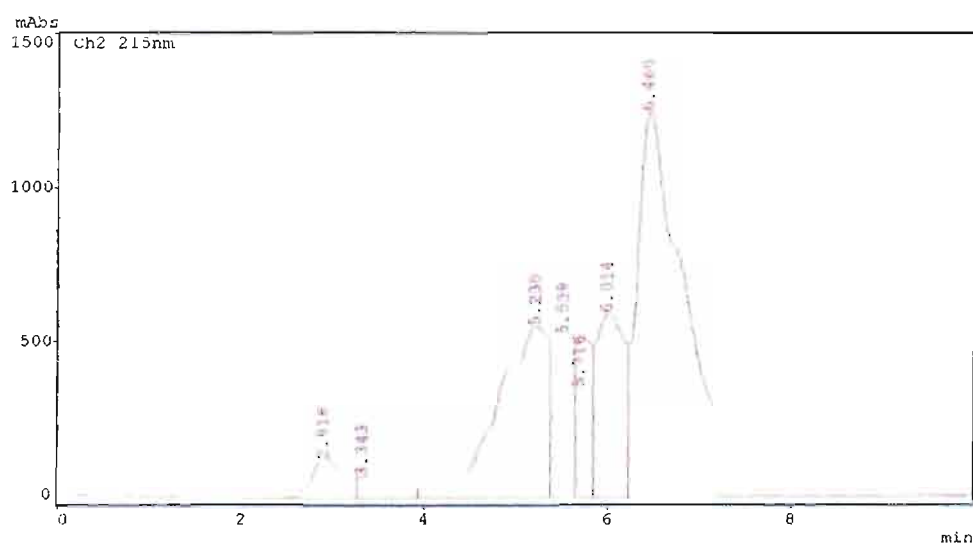
were the most active fractions in the 10% SPE fractions from the cold DCM root extract of *D. anomala* (batch I and II) from Lesotho (table 6.3).

Fractions 30, 40 and 50 from the SPE fractionation of the cold DCM root extract of *D. anomala* from Sterkspruit were as active against the D10 strain as those obtained from the cold DCM root extract of *D. anomala* from Lesotho. However, fraction 30 was more active than the one obtained from the cold DCM root extract of *D. anomala* from Lesotho. The results are shown in table 6.4 and figure 6.6.

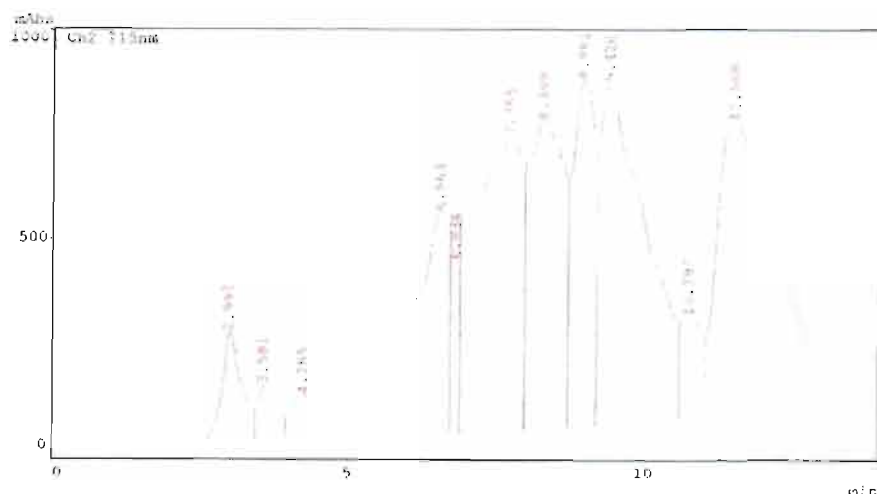


**Figure 6.6.** The dose response curves showing the antiplasmodial activity of 10% SPE fractions (fractions 30, 40 and 50) from the cold DCM root extract of *D. anomala* from Sterkspruit. Fractions 30 and 40 were screened at concentrations (50 µg/ml – 0.098 µg/ml) while fraction 50 was screened at concentrations (100 µg/ml – 0.195 µg/ml).

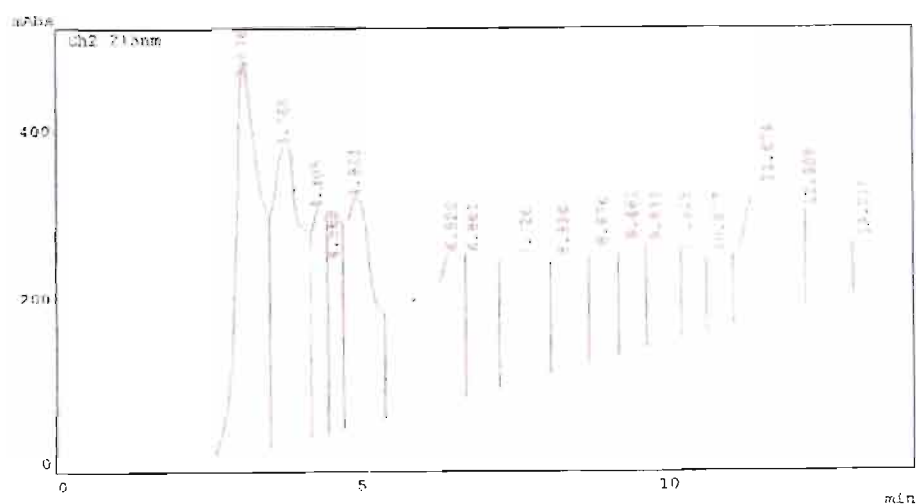
The HPLC profiles of the three fractions are shown in figures 6.7a - c and were similar to those obtained from batch I and II of the cold DCM root extract of *D. anomala* from Lesotho.



**Figure 6.7a.** The HPLC chromatogram of fraction 30 (4.5 mg/ml) from the cold DCM root extract of *D. anomala* from Sterkspruit. The fraction was run isocratically at 60% MeCN: H<sub>2</sub>O on a C18 Haisil reverse-phase semi-preparative column, at a flow rate of 2.0 ml/min and a detection wavelength of 215 nm. The injection volume was 150  $\mu$ l.



**Figure 6.7b.** The HPLC chromatogram of fraction 40 (4.5 mg/ml) from the cold DCM root extract of *D. anomala* from Sterkspruit. The fraction was run isocratically at 60% MeCN: H<sub>2</sub>O on a C18 Haisil reverse-phase semi-preparative column, at a flow rate of 2.0 ml/min and a detection wavelength of 215 nm. The injection volume was 150  $\mu$ l.



**Figure 6.7c.** The HPLC chromatogram of fraction 50 (4.5 mg/ml) from the cold DCM root extract of *D. anomala* from Sterkspruit. The fraction was run isocratically at 60% MeCN: H<sub>2</sub>O on a C18 Haisil reverse-phase semi-preparative column, at a flow rate of 2.0 ml/min and a detection wavelength of 215 nm. The injection volume was 150  $\mu$ l.

These results not only confirms that the roots collected in Sterkspruit are that of *D. anomala* but also indicate that they could be of the same subspecies (i.e. subspecies *anomala*). The findings could also be supported by the fact that Sterkspruit, which is in the north west of Eastern Cape Province is on the boundry with Free State Province and Lesotho. Mahale's Hoek (where the plant material was collected) is in the south west of Lesotho and is on the border with Sterkspruit. The distance between the two towns is about 42 km. The conditions, therefore, under which the plant is growing, would be similar.

## 6.5. CONCLUSION

- The fractionation of the cold DCM root extract of *D. anomala* from Lesotho and Sterkspruit, by SPE method, into 10% fractions did not result in the loss of antiplasmodial activity against D10 strain of *P. falciparum*. The most active fractions were fractions 30, 40 and 50. The activity was retained with the further fractionation of the cold DCM root extract into 5% and 1%. This showed that the fractionation process did not result in the breakdown of compounds and that the antiplasmodial activity of the cold DCM root extract was not due to synergism between the compounds.
- The SPE method was reproducible in obtaining 10% and 5% fractions from the cold DCM root extract of *D. anomala* from Lesotho. This was supported by the antiplasmodial activity and the consistency in the HPLC profiles of fractions 30, 40 and 50 (batch I and II).
- Although the 1% SPE fractions (fractions 35 – 55) did not result in pure compounds, their antiplasmodial activity against three CQR strains of *P. falciparum* (K1, RSA11 and FAC8) and against the CQS strain (D10) has indicated that these fractions contain antiplasmodial compounds that are both active against the CQS and CQR strains of *P. falciparum*. Some fractions were more active than others against the same strain (e.g. fractions 31 against D10 strain and fraction 32 against FAC8 strain) and



some showed similar activity against different strains (e.g. fraction 32 against RSA11 and FAC8 strains and fraction 34 against D10 and K1 strains).

- The roots of *D. anomala* collected from Lesotho and Sterkspruit could be the same as was observed in the similarity of the HPLC profiles of the cold DCM root extracts and their SPE fractions 30, 40 and 50.
- The roots of *D. anomala*, therefore, contain compounds that could act against both CQS and CQR strains of *P. falciparum*. Potential therefore exist for further development of these compounds into effective antimalarial drugs.

## ***CHAPTER 7***

### **THE ISOLATION, PURIFICATION, CHARACTERIZATION AND ANTIPLASMODIAL ACTIVITY OF COMPOUNDS MF4 and MF7A**

## 7.1. INTRODUCTION

Fractions 40 and 50 were the most active 10% SPE fractions (fraction 40:  $0.72 \pm 0.20 \mu\text{g/ml} \leq \text{IC}_{50} \leq 0.63 \pm 0.045 \mu\text{g/ml}$  and fraction 50:  $0.63 \pm 0.0 \mu\text{g/ml} \leq \text{IC}_{50} \leq 0.54 \pm 0.11 \mu\text{g/ml}$ ) (Chapter 5, table 5.4) obtained from the fractionation of the cold DCM root extract of *D. anomala* from Lesotho. Two compounds, MF4 and MF7A, were isolated from these fractions.

## 7.2. METHODS

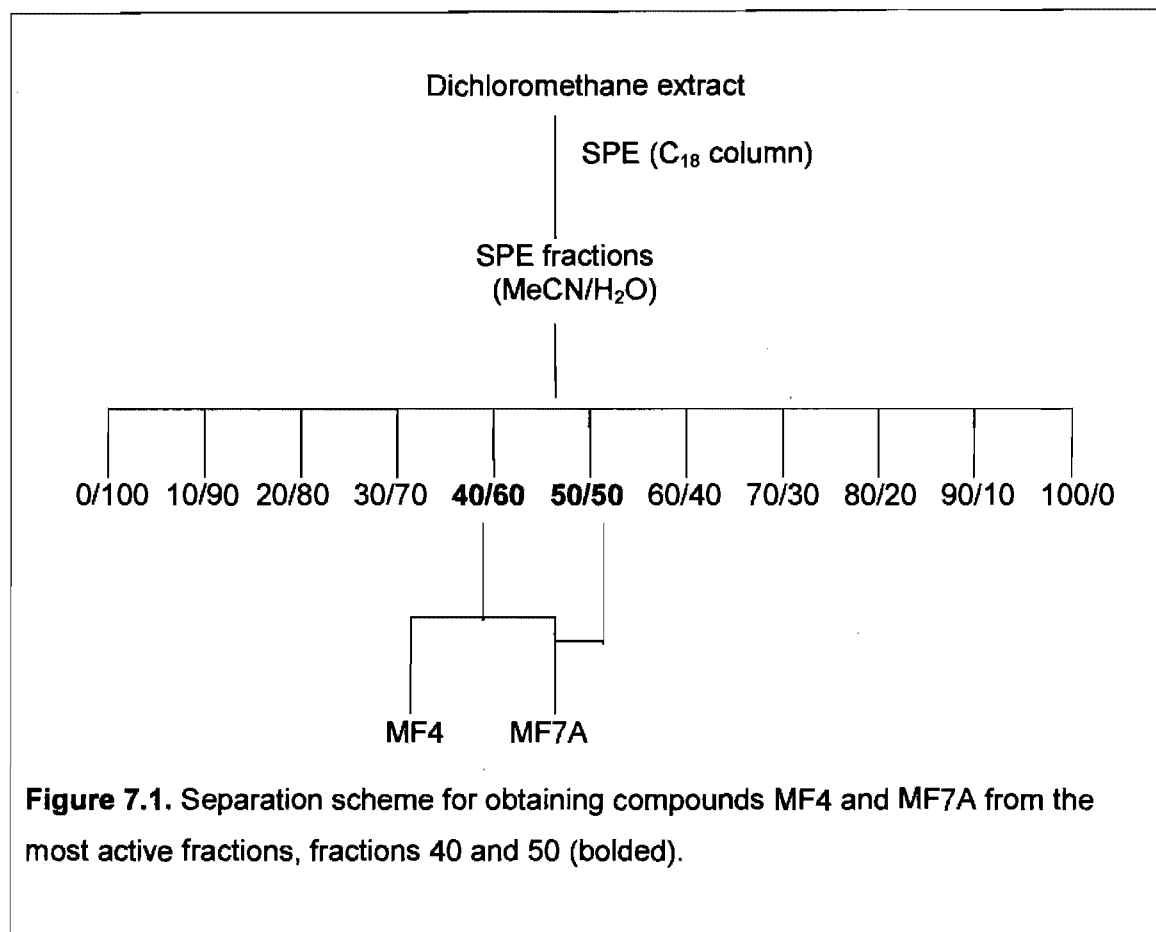
Compounds, MF4 and MF7A, were both isolated from fraction 40 and only compound MF7A was isolated from fraction 50 on semi-preparative HPLC. Although MF4 was collected as a single peak and MF7A collected separately from the other peaks, they were not pure and therefore were purified further on analytical HPLC.

Compounds MF4 and MF7A were tested for antiplasmodial activity against CQS (D10) and CQR (K1 and RSA11) strains of *P. falciparum* and their cytotoxicity was determined on the CHO cells.

The chemical structure of compound MF7A and the partial chemical structure of compound MF4 were identified by NMR and Mass Spectrometry analysis. The structures were elucidated by MS,  $^1\text{H}$  and  $^{13}\text{C}$  NMR and confirmed by 2D NMR methods (COSY, NOESY, HSQC and HMQC). NMR analyses were carried out on a Varian Inova 600 NMR spectrometer. The mass spectra were obtained using APCI in the positive ion mode on API 2000 LC/MS/MS mass spectrometer and the HRMS on Waters API Q-TOF Ultima. A detailed report of the methods used is in Chapter 4.

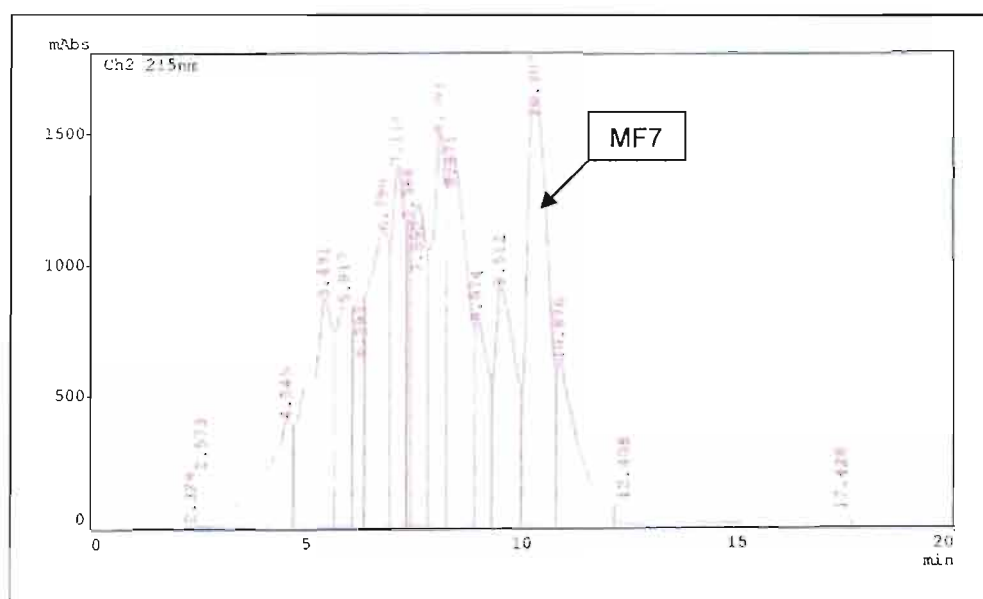
## 7.3 RESULTS AND DISCUSSION

### 7.3.1. The Isolation of Compounds MF4 and MF7A

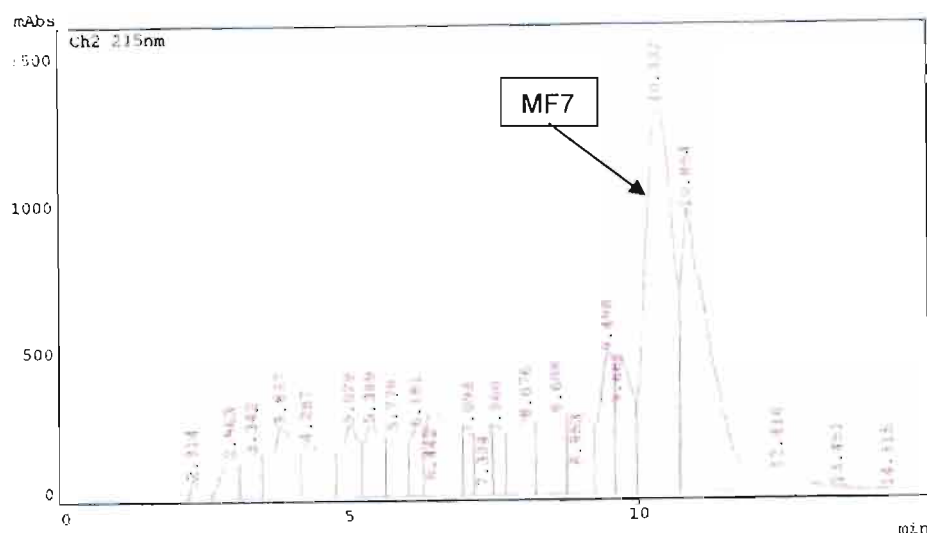


**Figure 7.1.** Separation scheme for obtaining compounds MF4 and MF7A from the most active fractions, fractions 40 and 50 (bolded).

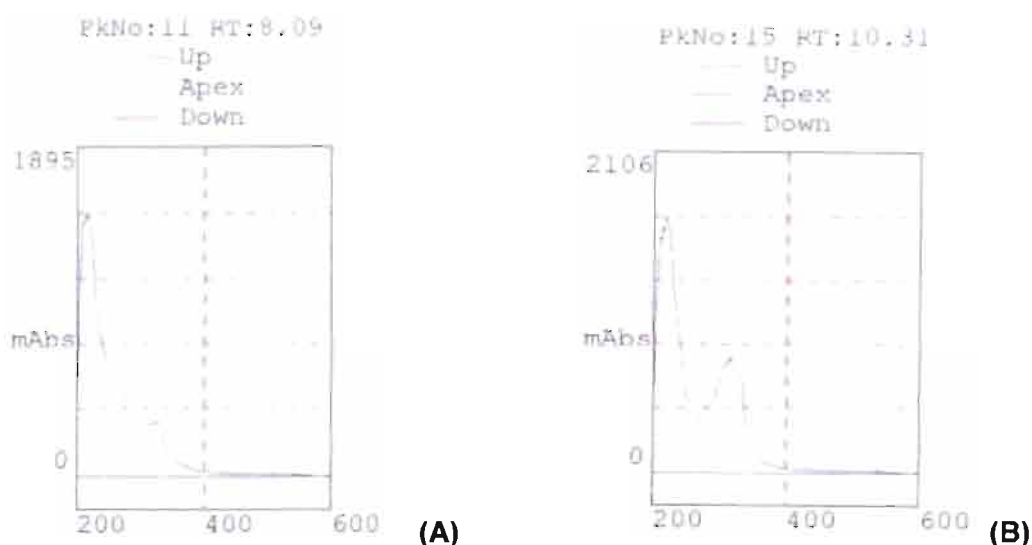
Fractions 40 and 50 were first run with a gradient elution of 50% - 70% MeCN: H<sub>2</sub>O at a flow rate of 3 ml/min. The fractions (5 mg/ml) were loaded on a C18 Haisil 100 reverse-phase semi-preparative column (250 x 10 mm, 5 µm), the injection volume was 260 µl and the detection wavelength was set at 215 nm. The HPLC chromatograms are shown in figures 7.2a and 7.2b.



**Figure 7.2a.** Reverse phase semi-preparative HPLC for fraction 40 (5 mg/ml) run on a C18 Haisil 100 column (250 x 10 mm, 5  $\mu$ m) at a gradient of 50% - 70% MeCN: H<sub>2</sub>O. MF4 eluted at 8.091 minutes and MF7 at 10.307 minutes. The flow rate was 3 ml/min, the injection volume was 260  $\mu$ l and the detection wavelength was set at 215 nm.

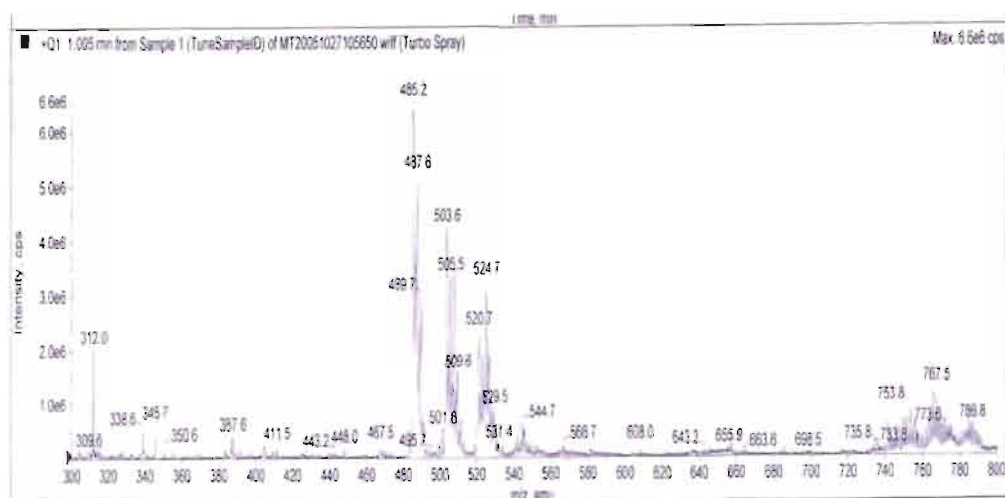


**Figure 7.2b.** Reverse phase semi-preparative HPLC for fraction 50 (5 mg/ml) run on a C18 Haisil 100 column (250 x 10 mm, 5  $\mu$ m) at a gradient of 50% - 70% MeCN: H<sub>2</sub>O. MF7 eluted at 10.332 minutes. The flow rate was 3 ml/min, the injection volume was 260  $\mu$ l and the detection wavelength was set at 215 nm.

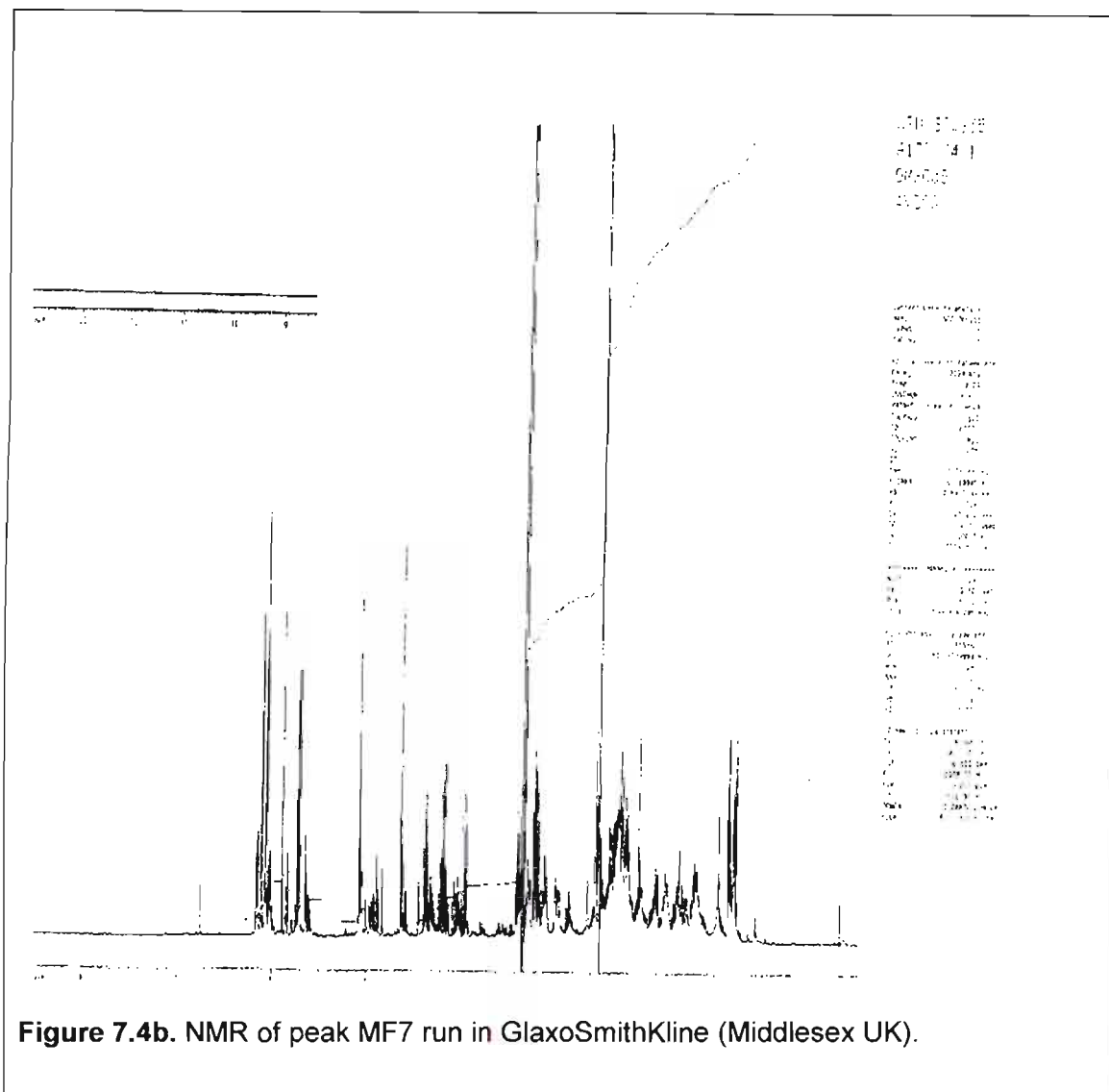


**Figure 7.3.** The UV spectra of MF4 (A) and MF7 (B).

The NMR spectrum of peak MF7 was poorly resolved and its Mass Spectrometry showed molecular ions ( $M + H$ ) at  $m/z$  485, 487, 489 (2.2: 1.7: 1) and others at  $m/z > 500$  as shown in figure 7.4a. The major molecular ion in both samples was at  $m/z$  485. This showed that the MF7 sample was not a pure compound and explained that the poor resolution of the NMR spectrum of MF7 was due to overlapping signals (figure 7.4b).



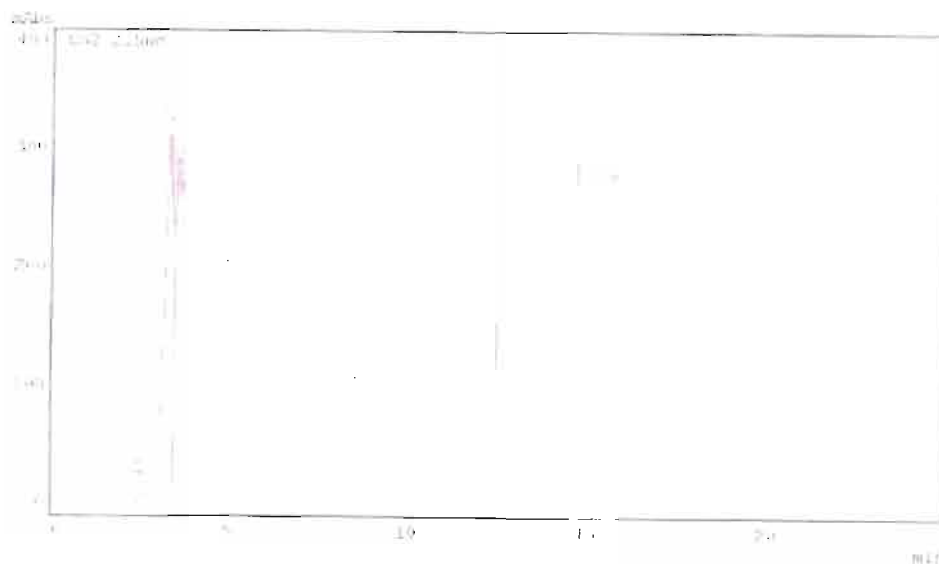
**Figure 7.4a.** LC/MS of peak MF7.



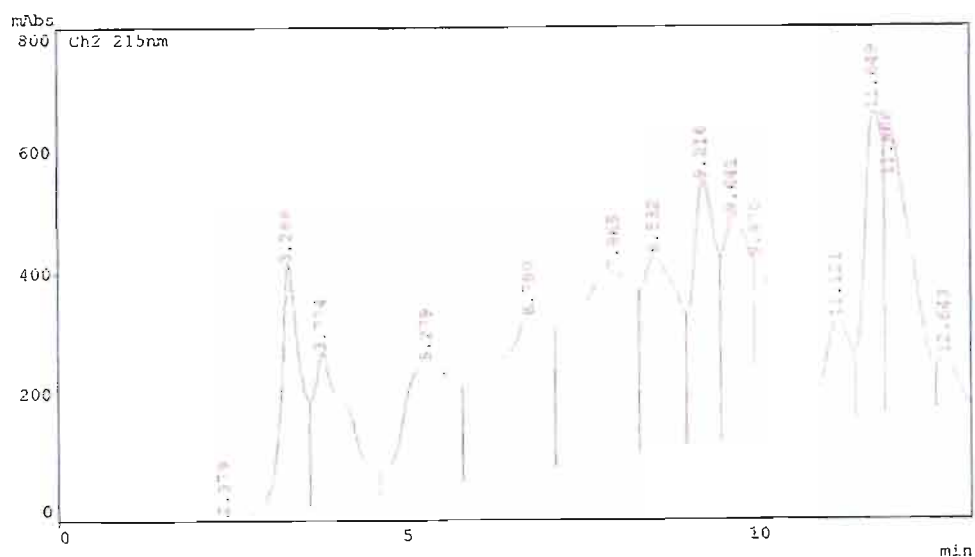
**Figure 7.4b.** NMR of peak MF7 run in GlaxoSmithKline (Middlesex UK).

### 7.3.2. Separation of components in peak MF7

Fraction 40 was eluted at isocratic elutions of 50%, 60% and 70% MeCN: H<sub>2</sub>O. The fraction was loaded at a concentration of 4.5 mg/ml on a Haisil semi-preparative column (250 x 10 mm, 5  $\mu$ m) and the compounds eluted at a flow rate of 2.5 ml/min. The injection volume was 250  $\mu$ l and the detection wavelength was at 215 nm. Peak MF7 separated into two peaks with the isocratic elution of 60% MeCN: H<sub>2</sub>O. The HPLC chromatograms are shown in figures 7.5a, 7.5b and 7.5c.

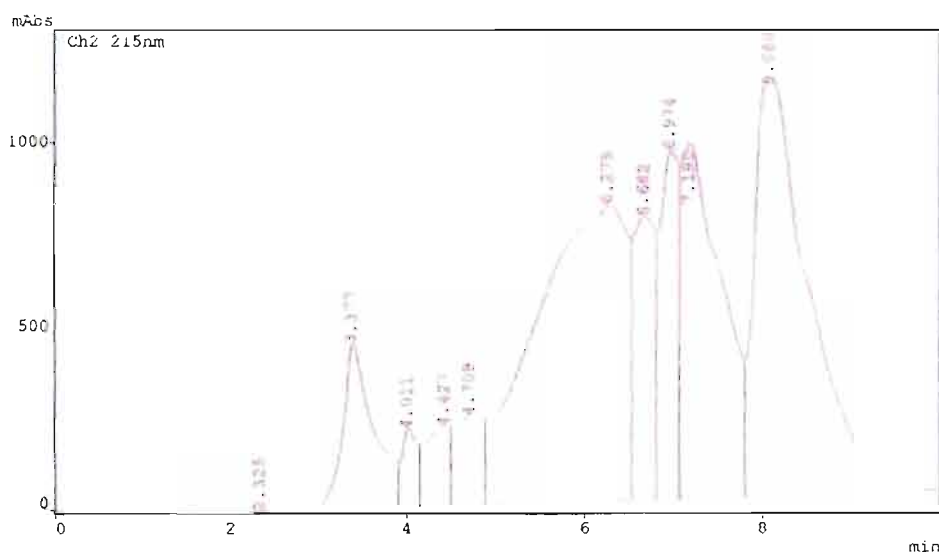


**Figure 7.5a.** Reverse phase semi-preparative HPLC for fraction 40 (4.5 mg/ml) run on a Haisil column (250 x 10 mm, 5  $\mu$ m), at an isocratic elution of 50% MeCN in H<sub>2</sub>O. The flow rate was 2.5 ml/min, the injection volume was 250  $\mu$ l and the detection wavelength was set at 215 nm. Peak MF7 eluted at 21.297 minutes as a single peak, showing a small degree of separation.



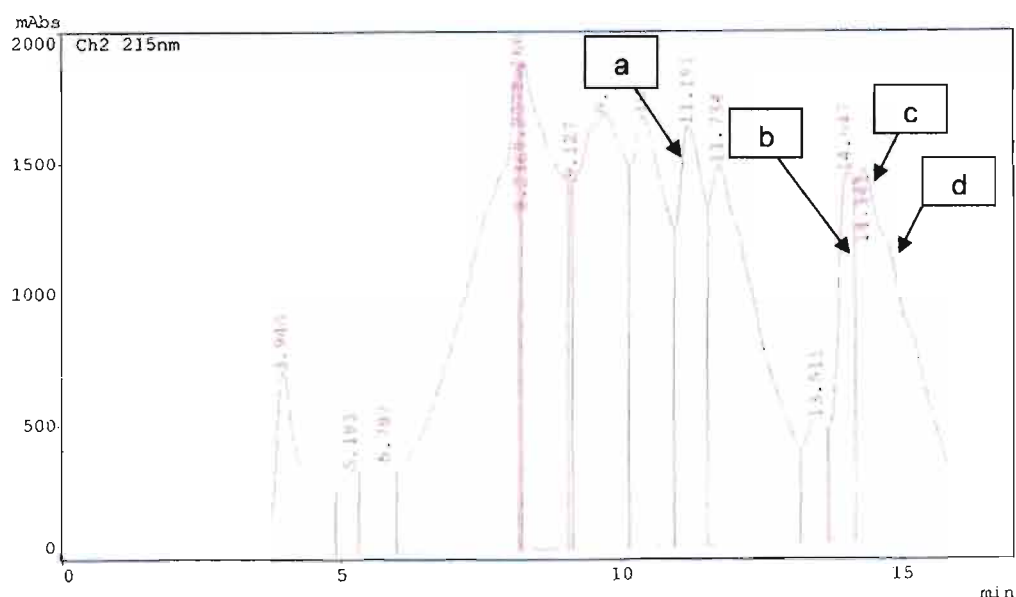
**Figure 7.5b.** Reverse phase semi-preparative HPLC for fraction 40 (4.5 mg/ml) run on a Haisil column (250 x 10 mm, 5  $\mu$ m), at an isocratic elution of 60% MeCN:H<sub>2</sub>O. The flow rate was 2.5 ml/min, the injection volume was 250  $\mu$ l and the detection wavelength was set at 215 nm. Peak MF7 separated into two peaks, which eluted at 11.649 minutes and 11.866 minutes, respectively.



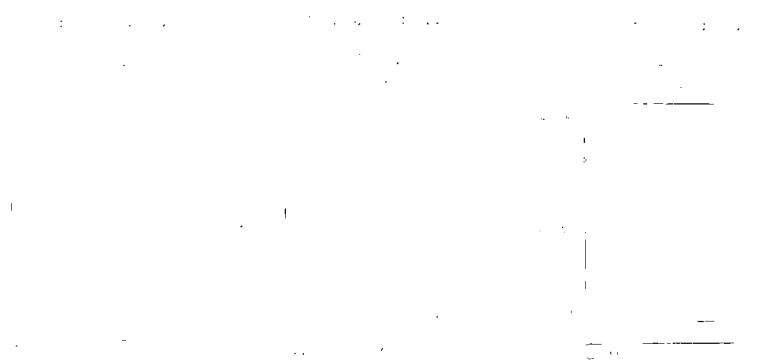


**Figure 7.5c.** Reverse phase semi-preparative HPLC for fraction 40 (4.5 mg/ml) run on a Haisil column (250 x 10 mm, 5  $\mu$ m), at an isocratic elution of 70% MeCN: H<sub>2</sub>O. The flow rate was 2.5 ml/min, the injection volume was 250  $\mu$ l and the detection wavelength was set at 215 nm. Peak MF7 is eluting at 8.080 minutes as a broad single peak.

Since the isocratic elution of fraction 40 at 60% MeCN: H<sub>2</sub>O showed the separation of peak MF7 into two peaks, two parameters were changed to determine if peak MF7 would separate further. The flow rate and the injection volume were reduced to 2.0 ml/min and 150  $\mu$ l, respectively. The separation of MF7 into three peaks was achieved by an isocratic elution at 60% MeCN: H<sub>2</sub>O and the three peaks were designated MF7A, MF7B and MF7C with MF7A as the major peak (figures 7.5d).



**Figure 7.5d.** Reverse phase semi-preparative HPLC for fraction 40 (4.5 mg/ml) run at an isocratic elution of 60% MeCN:H<sub>2</sub>O. The flow rate was 2.0 ml/min, the injection volume was 150  $\mu$ l and the detection wavelength was set at 215 nm. The HPLC chromatogram showed the separation of peak MF7 into three peaks. Peak MF4 (a) eluted at 11.191 minutes and peaks MF7A (b), MF7B (c) and MF7C (d) at 14.047, 14.341 and  $\approx$  15 minutes, respectively.

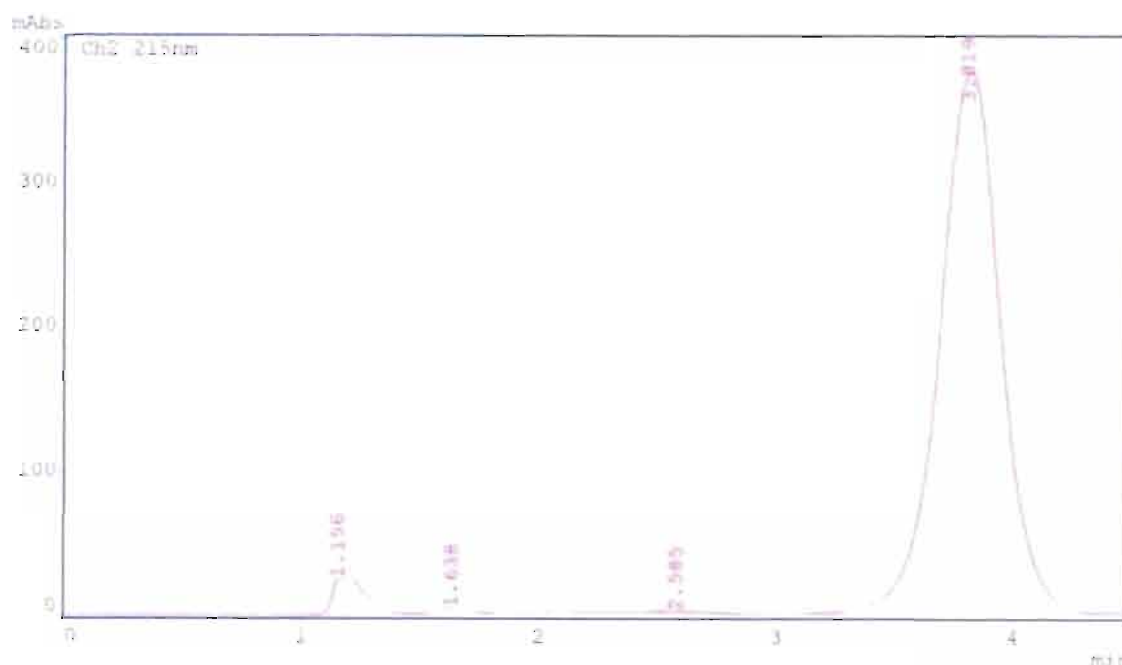


**Figure 7.5e.** The UV spectra of MF7A, MF7B and MF7C.

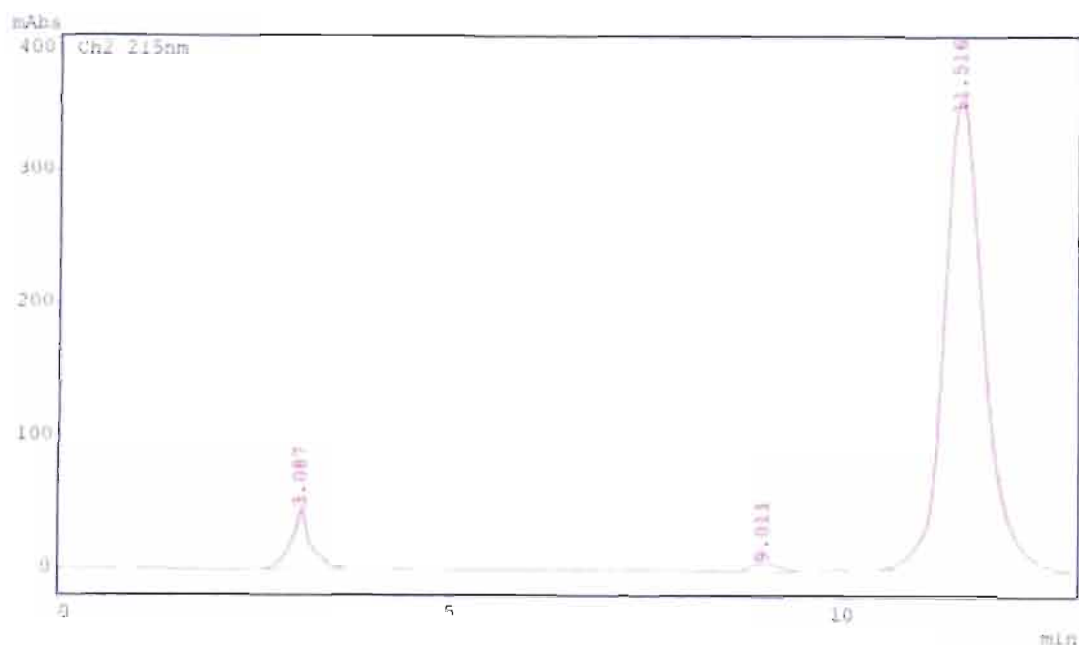
The UV spectra of MF7A, MF7B and MF7C were similar (figure 7.5e), which explained the co-elution of these compounds. MF7A was present in higher amounts as compared to MF7B and MF7C and was easier to collect.

### 7.3.3. Purification of Compounds MF4 and MF7A

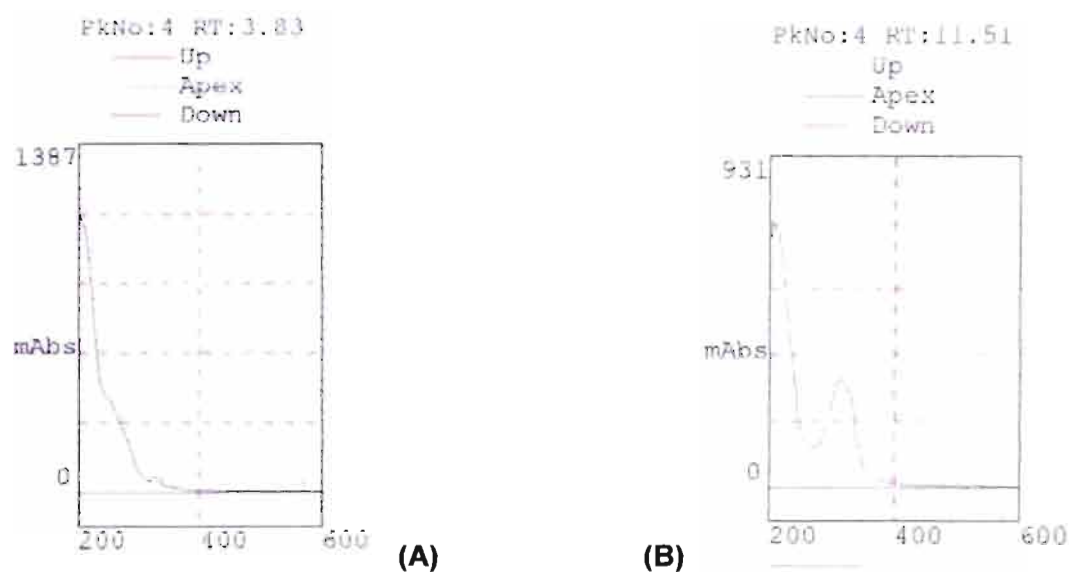
Compounds MF4 and MF7A were purified further on analytical HPLC. The compounds were loaded at a concentration of 2 mg/ml on the Haisil analytical columns (250 x 4.6 mm, 5  $\mu$ m for MF7A and 150 x 4.6 mm, 5  $\mu$ m for MF4) and eluted isocratically at 60% MeCN: H<sub>2</sub>O. The flow rate was 1 ml/min, the injection volume was 150  $\mu$ l and the compounds were monitored at 215 nm. The yields of the compounds were very low [MF4 = 0.17 mg (0.38%) and MF7A = 0.08 mg (0.18%) from 45 mg of fraction 40]. Figures 7.6, 7.7 and 7.8 show the HPLC chromatograms and UV spectra of compounds MF4 and MF7A.



**Figure 7.6.** The analytical HPLC chromatogram of compound MF4 (3.819 minutes). The compound was run on a Haisil analytical column (150 x 4.6 mm, 5  $\mu$ m) at an isocratic run of 60% MeCN: H<sub>2</sub>O. The flow rate was 1 ml/min, the injection volume was 150  $\mu$ l and the compound was monitored at 215 nm.



**Figure 7.7.** The analytical HPLC chromatogram of compound MF7A (11.516 minutes). The compound was run on a Haisil analytical column (250 x 4.6 mm, 5  $\mu$ m) at an isocratic run of 60% MeCN: H<sub>2</sub>O. The flow rate was 1 ml/min, the injection volume was 150  $\mu$ l and the compound was monitored at 215 nm.



**Figure 7.8.** The UV spectra of compounds MF4 (A) and MF7A (B).

### 7.3.4. Mass Spectrometry

The mass spectra were recorded on an API 2000 LC/MS/MS mass spectrometer.

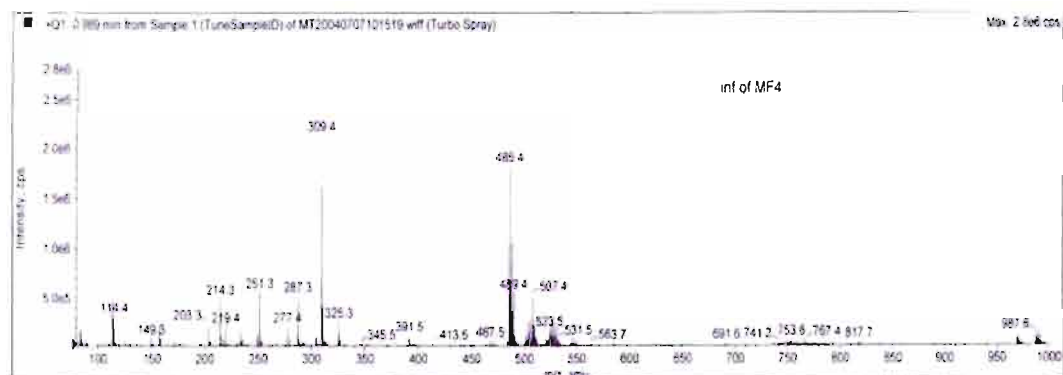


Figure 7.9a. LC-MS of compound MF4.

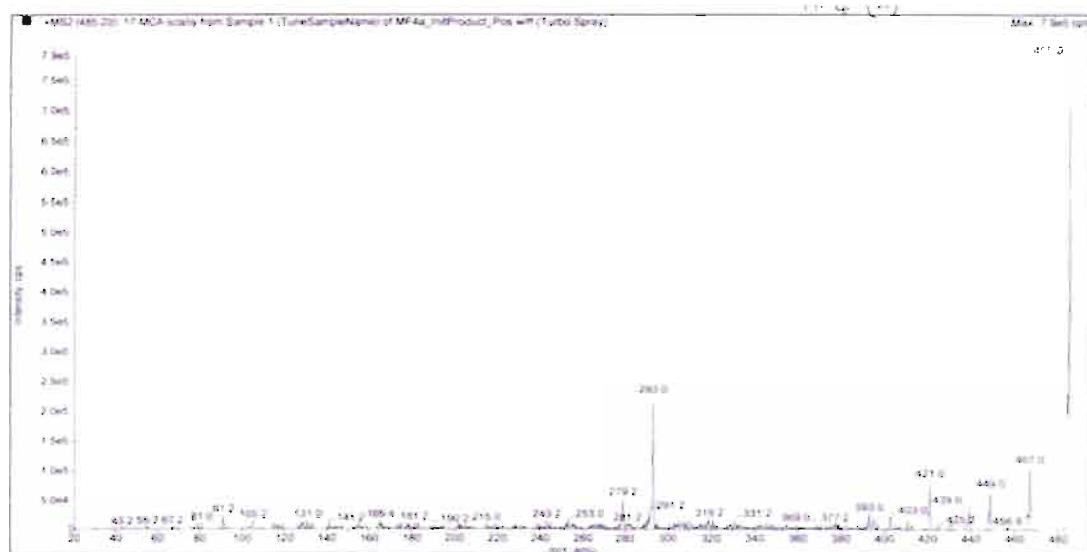


Figure 7.9b. APCI of compound MF4.

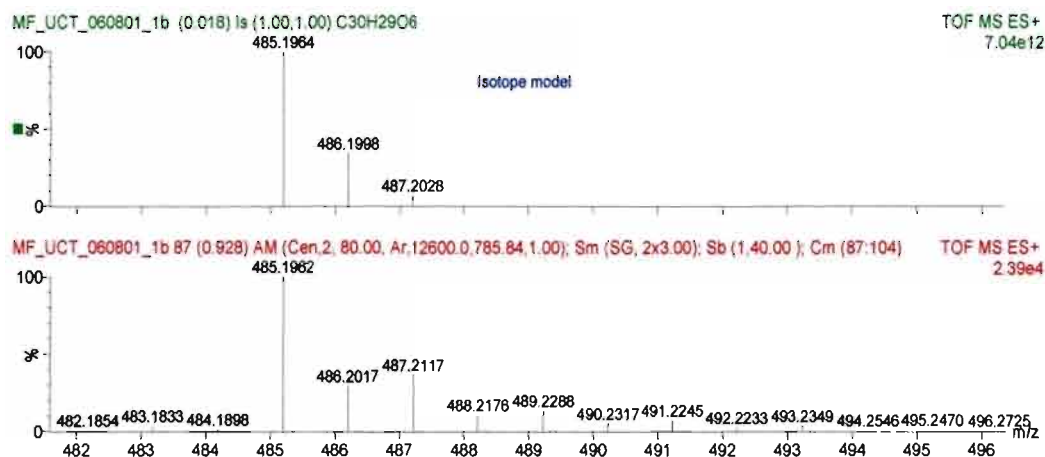


Figure 7.9c. ESI-HRMS of compound MF4.

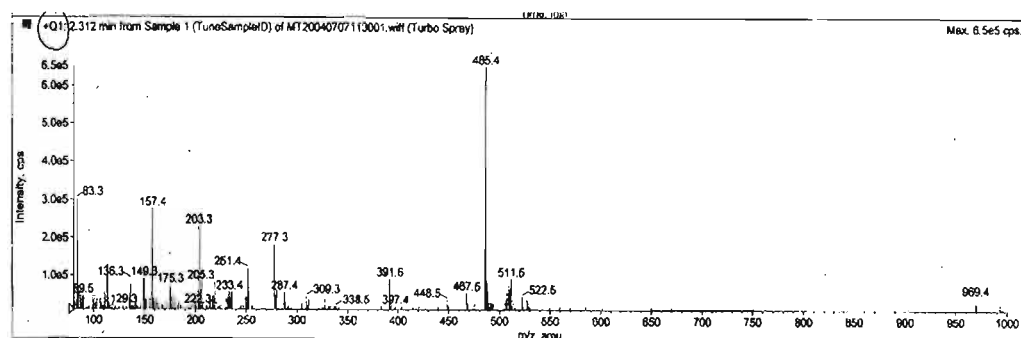


Figure 7.10a. LC-MS of compound MF7A.

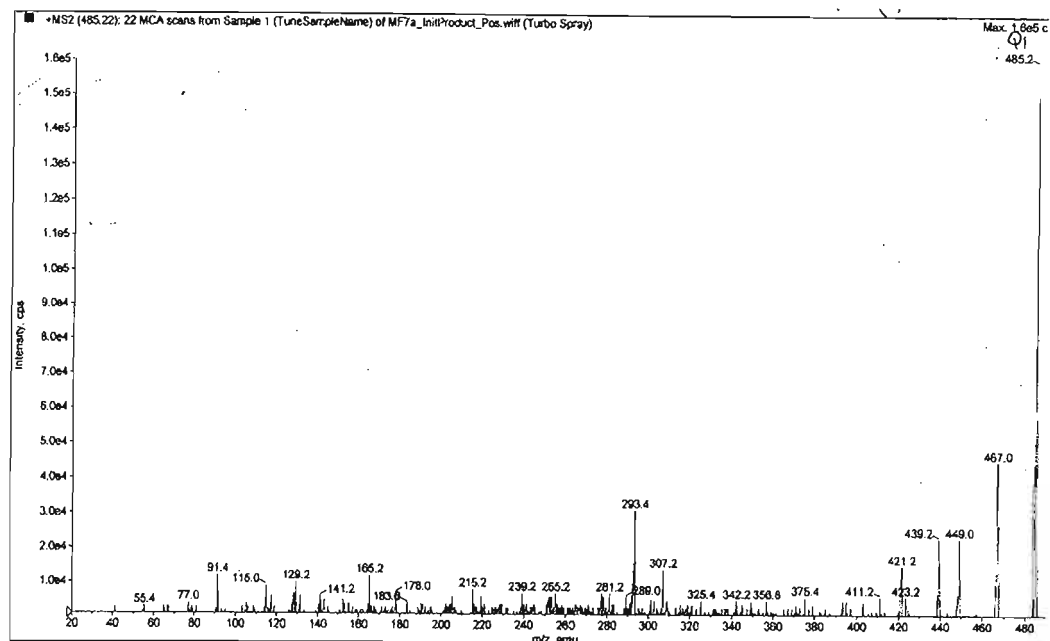


Figure 7.10b. APCI of compound MF7A.

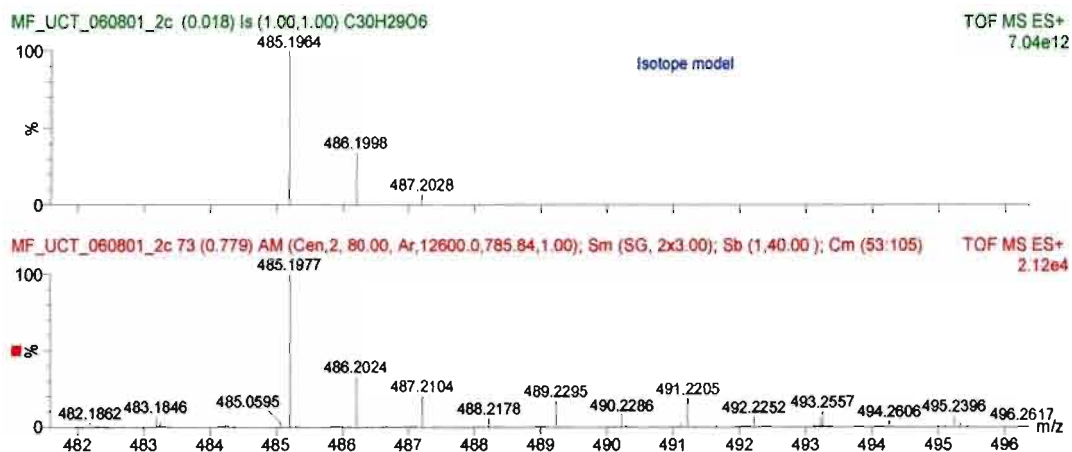


Figure 7.10c. ESI-HRMS of compound MF7A.

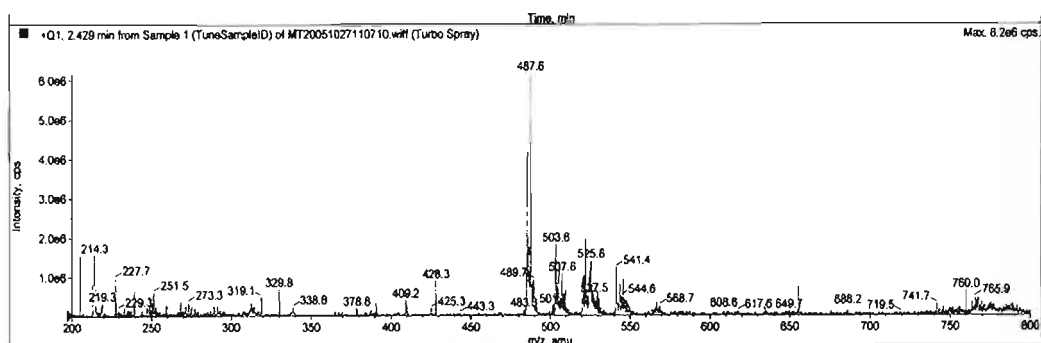


Figure 7.11a. LC-MS of peak MF7B.

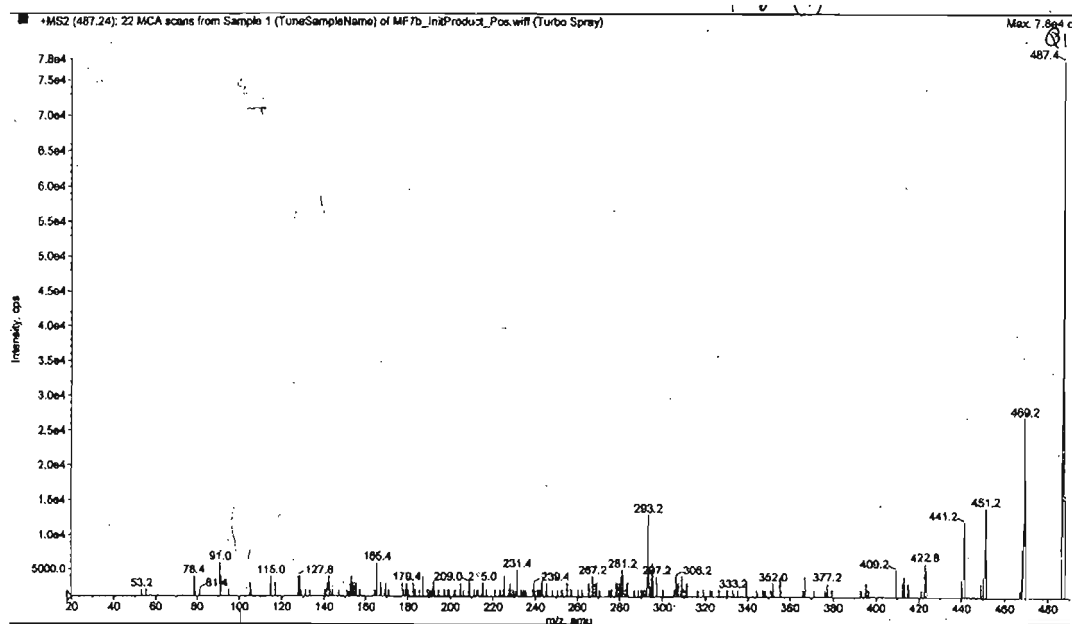


Figure 7.11b. APCI of peak MF7B.



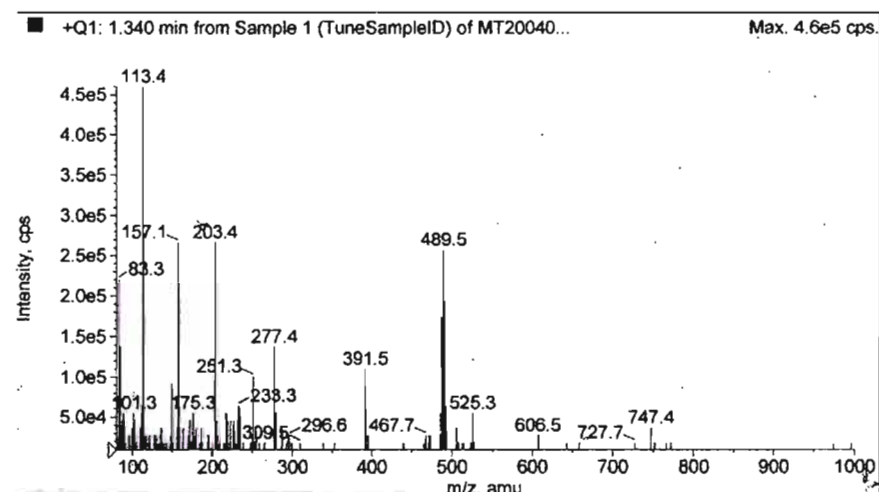


Figure 7.12a. LC-MS of peak MF7C.

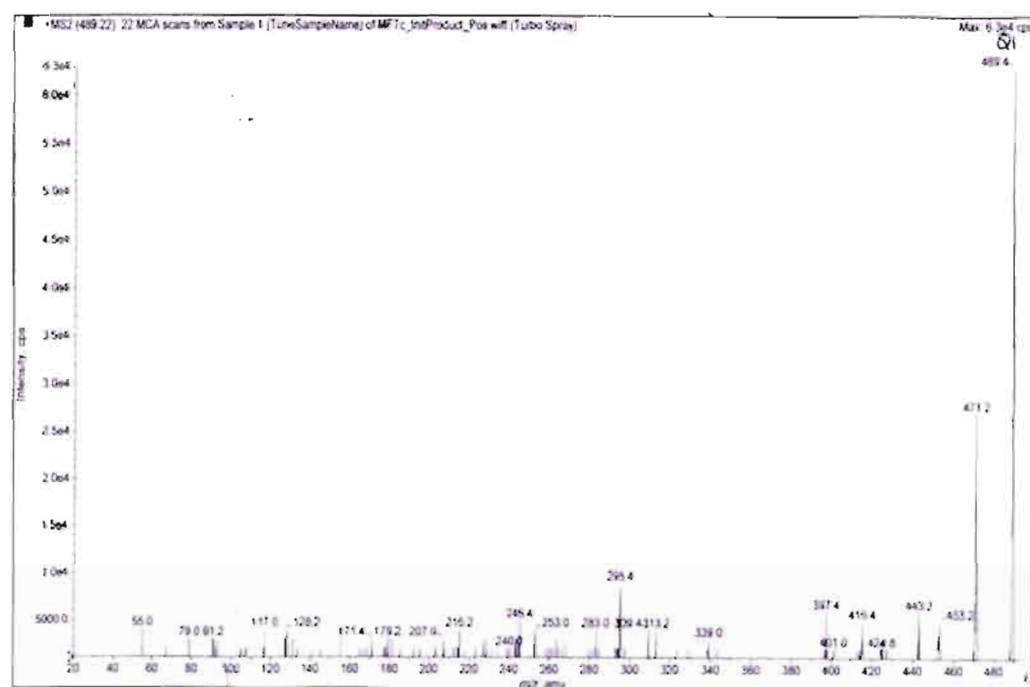


Figure 7.12b. APCI of peak MF7C.

Compounds MF4 and MF7A showed the experimental mass  $[M + H]^+$  of 485.1962 and 485.1977, respectively, and the calculated mass of 485.1964 for both. The mass was consistent with the molecular formula of  $(C_{30}H_{29}O_6)$ , therefore, the molecular formulae of compounds MF4 and MF7A were  $(C_{30}H_{28}O_6)$ . The elemental composition of compounds MF4 and MF7A are shown in Appendix E. Compounds MF4 and MF7A had the same molecular mass, ion mass fragmentation patterns and molecular formulae but they eluted at different retention times on the HPLC (figure 7.5a). This strongly indicated that the two compounds are structural isomers.

Compound MF7A and peaks MF7B and MF7C had molecular ion masses  $m/z$  at  $[M + H]^+$  which differed by two amu (485, 487 and 489, respectively) (figures 7.10a, 7.11a and 7.12a). Their ion mass fragmentation patterns also differed by two amu (figures 7.10b, 7.11b and 7.12b), indicating the hydrogenation of the double bond/s. This showed that MF7B and MF7C have similar chemical structures to compound MF7A (figure 7.17) and is further supported by the similarity in the UV spectra of these peaks. The level of purity and quantity of MF7B and MF7C did not allow further purification for structural elucidation using NMR.

A similar observation was made with peak MF8, which eluted after peak MF7C (Appendix E). Its molecular ion mass  $m/z$  at  $[M + H]$  was 491, indicating a difference of 2 amu from MF7C (489) and its ion mass fragmentation pattern also differed from that of MF7C by 2 amu. This means that compound MF7A, peaks MF7B, MF7C and MF8 differed from each other by 2 amu, indicating the level of hydrogenation bond/s and similar chemical structures. Peaks MF5 and MF6, which eluted after peak MF4 also had molecular ion masses which differed by 2 amu (524 for MF5 and 526 for MF6B and 528 for MF6E) (Appendix E). Their ion mass fragmentation patterns also differed by 2 amu, indicating similar chemical structures.

Another observation was that peaks MF5 and MF7C had common mass ions in their ion mass fragmentation patterns and similarly for peaks MF6B and MF8,

and MF7B and MF8 (Appendix E). Due to the difficulty in purifying peaks MF6B, MF6E and MF8 and obtaining sufficient amounts and the instability of peak MF5 (Chapter 4, p. 70) which could have been to absorption of moisture and oxidation, the isolation of pure compounds could not be achieved.

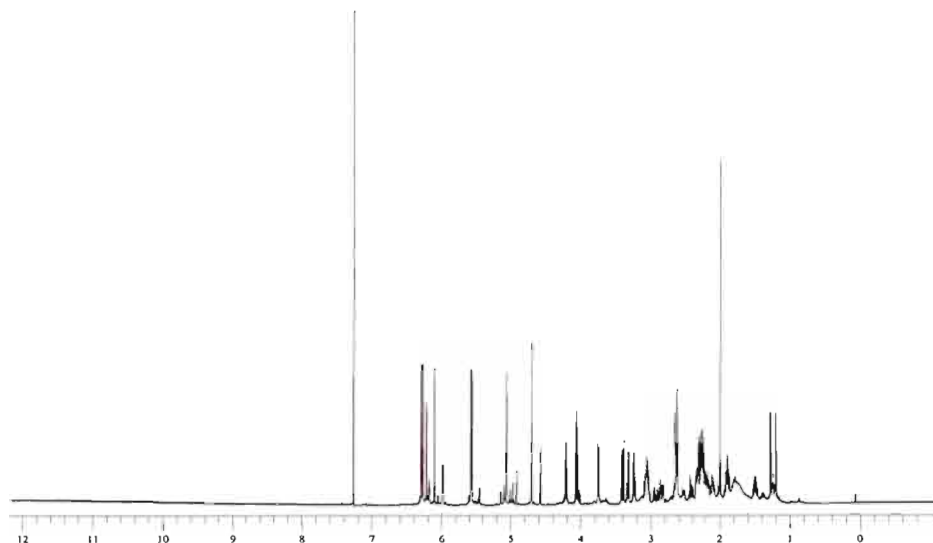
These results have shown that the roots of *D. anomala* contain compounds with similar chemical structures and which are possible dimeric sesquiterpenes.

### 7.3.5. Nuclear Magnetic Resonance (NMR)

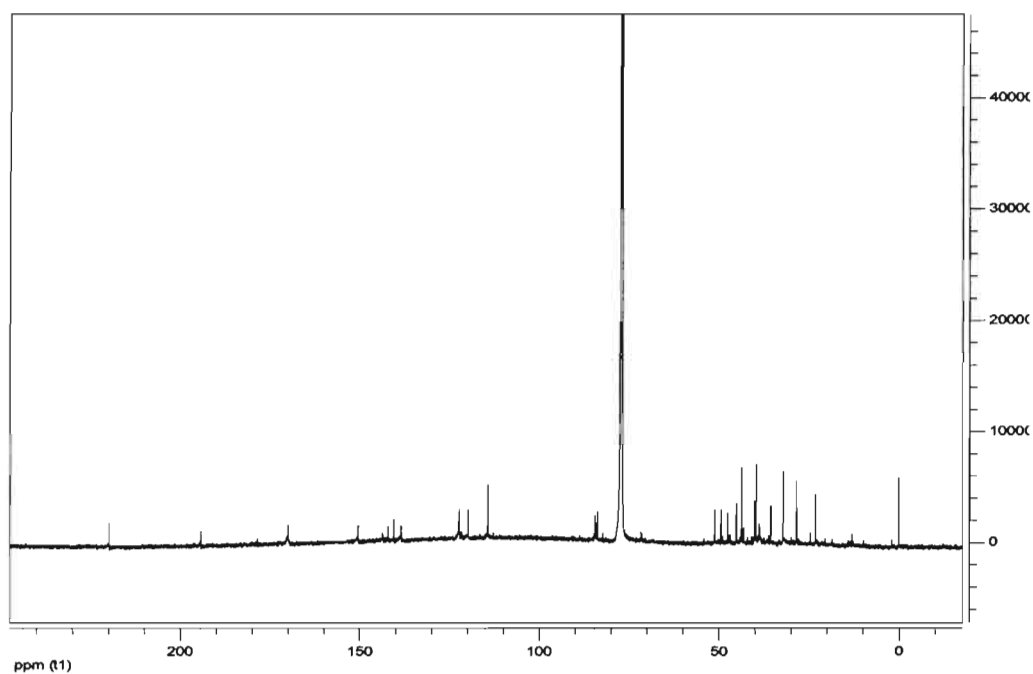
The NMR spectra ( $^1\text{H}$ ,  $^{13}\text{C}$ , COSY, NOESY, HSQC, HMQC and DEPT) were recorded on a Varian Inova 600 spectrometer at 25°C in  $\text{CDCl}_3$ . The  $^1\text{H}$  and  $^{13}\text{C}$  spectra were recorded at a frequency of 600 MHz and 150 MHz, respectively. All the chemical shifts ( $\delta$ ) are reported in parts per million (ppm) relative to TMS ( $\delta = 0$ ) as internal standard.

#### 7.3.5.1. NMR spectra of Compound MF7A

##### 7.3.5.1.1. $^1\text{H}$ -NMR spectrum



**Figure 7.13.**  $^1\text{H}$ -NMR spectrum of compound MF7A ( $\text{C}_{30}\text{H}_{28}\text{O}_6$ ).

**7.3.5.1.2.  $^{13}\text{C}$ -NMR spectrum**

**Figure 7.14.**  $^{13}\text{C}$ -NMR spectrum of compound MF7A ( $\text{C}_{30}\text{H}_{28}\text{O}_6$ ).

## 7.3.5.1.3. Correlation Spectroscopy (COSY)

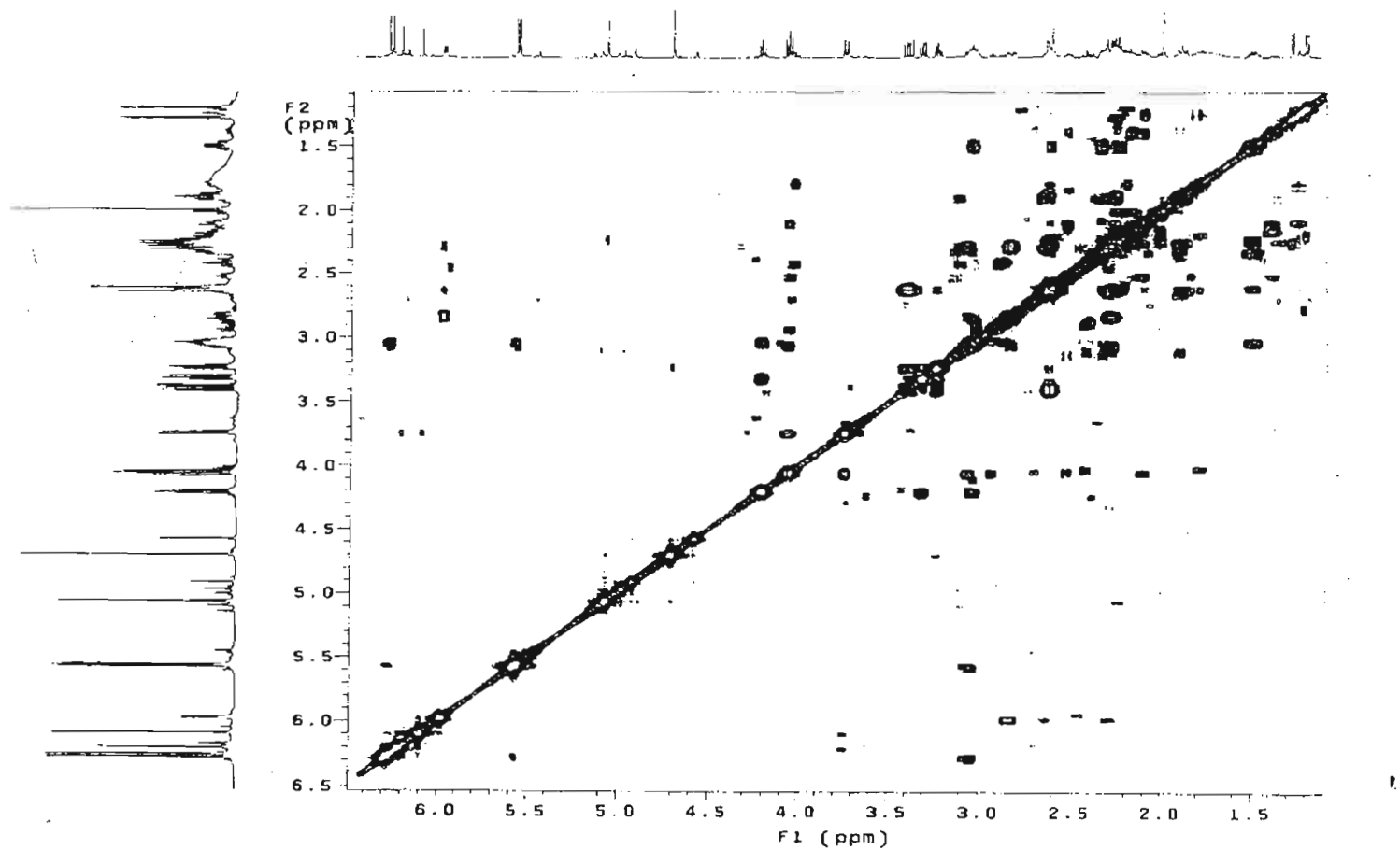


Figure 7.15. COSY spectrum of compound MF7A ( $C_{30}H_{28}O_6$ ).

## 7.3.5.1.4. 2D-NMR spectrum (HSQC)

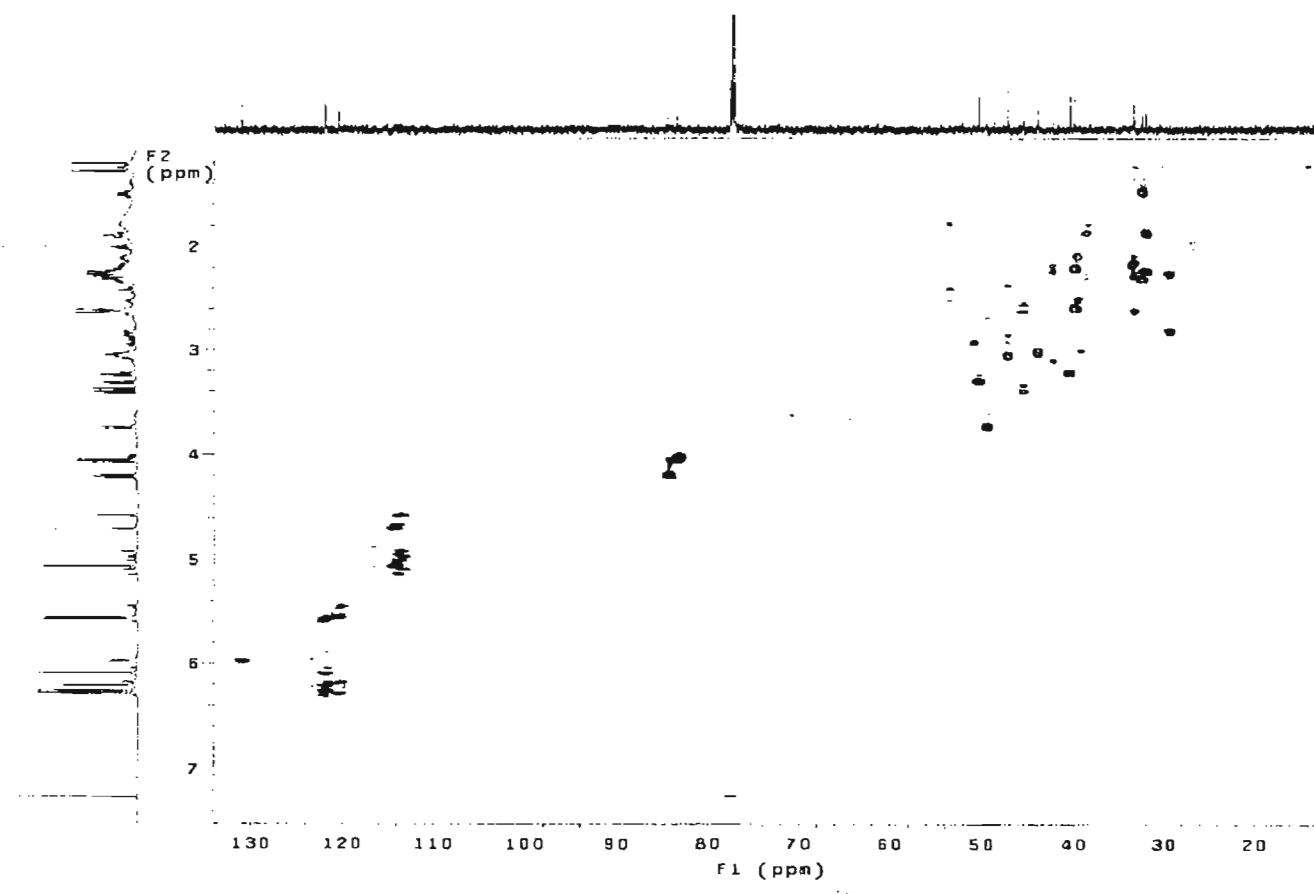
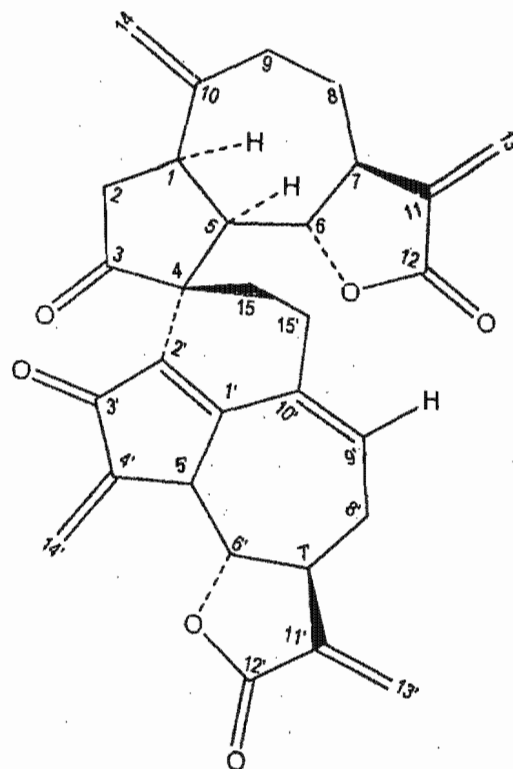


Figure 7.16. HSQC spectrum of compound MF7A (C<sub>30</sub>H<sub>28</sub>O<sub>6</sub>).



**Figure 7.17.** Chemical structure of compound MF7A ( $C_{30}H_{28}O_6$ ) isolated from fractions 40 and 50.

**Table 7.1.**  $^1\text{H}$  and  $^{13}\text{C}$ -NMR spectral data for compound MF7A (600 MHz and 150 MHz,  $\text{CDCl}_3$ ).

H Position	Chemical shift ( $\delta$ ) (J in Hz)	HSQC	HMQC	COSY	NOESY
1	3.24 m	40.2 d	C-2, C-3, C-4, C-5, C-10, C-14	H2 $\alpha$ , H2 $\beta$ , H5	H1
2 $\alpha$	2.64 m	45.3 t	C-1, C-3, C-4, C-5, C-10	H1	
2 $\beta$	3.40 dd (18.9, 10.2)	-	C-3, C4, C-5, C-10	H1	
3	-	220.0 s			
4	-	51.9 s			H1, H7
5	3.32 dd (10.2, 8.4)	50.1 d	C-2', C-4, C-6, C-15	H6	
6	4.21 dd (10.2, 9.6)	84.2 d	C-4, C-8	H7	
7	3.05 m	43.5 d		H8 $\beta$	
8 $\alpha$	1.91 m	31.9 t	C-6	H9 $\alpha$ , H8 $\beta$	
8 $\beta$	1.50 m	-		H8 $\alpha$	
9 $\alpha$	2.61 m	39.6 t	C-1, C8, C10, C-14	H9 $\beta$	
9 $\beta$	2.31 m	-	C-1, C8, C10, C-14		
10	-	150.7 s			
11	-	138.7 s			
12	-	170.7 s			
13a	6.26 d (3.3)	121.6 t	C-7, C-11, C-12	H13b	
13b	5.58 d (3.3)	-	C-7, C-11, C-12	H13a	
14a	5.07 s	114.3 t	C-1, C-10		
14b	4.7 s	-	C-1, C-10		
15 $\alpha$	2.25 m	31.6	C-3, C-4, C-5, C-15'	15 $\beta$	
15 $\beta$	1.88 m		C-4, C-5, C-15'	15 $\alpha$	
1'	-	158.5 s			
2'	-	144.8 s			
3'	-	193.1 s			
4'	-	141.2 s			
5'	3.75 d (10.8)	49.3 d	C-1', C-2', C-4'	H6'	H7'
6'	4.06 dd (10.8, 9.6)	83.2 d	C-8'	H5', H7'	



Table 7.1. cont.

H Position	Chemical shift ( $\delta$ ) (J in Hz)	HSQC	HMQC	COSY	NOESY
7'	3.04 m	46.9 d		H6'	
8' $\alpha$	2.81-2.97 m	29.2 d		H7', H8 $\beta$	
8' $\beta$	2.81-2.97 m	-		H8 $\alpha$ , H9'	
9'	5.98 d (6.6)	130.9 d	C-1', C-7', C-15'		
10'	-	134.4 s			
11'	-	138.2 s			
12'	-	169.5 s			
13'a	6.29 d (3.3)	120.2 t	C-7', C-11', C-12'	H13'b	
13'b	5.56 d (3.3)	-	C-7', C-11', C-12'	H13'a	
14'a	6.21 s	121.7 t	C-3', C-4', C-5'		
14'b	6.09 s	-	C-3', C-4', C-5'		
15' $\alpha$	2.61 m	32.9	C-15	H15' $\alpha$ , H15 $\alpha$	
15' $\beta$	2.18 m	-	C-15	H15' $\beta$ , H15 $\beta$	

### 7.3.5.1.5. Proton and Carbon Assignments for Compound MF7A

The complete proton and carbon resonances were achieved from the 1D and 2D-NMR spectra (table 7.1). The  $^1\text{H}$ -NMR showed two sets of signals for exomethylene protons at  $\delta = 6.26$  [d,  $J = 3.3$  Hz, H-13a],  $\delta = 5.58$  [d,  $J = 3.3$  Hz, H-13b],  $\delta = 6.29$  [d,  $J = 3.3$ , H-13'a],  $\delta = 5.56$  [d,  $J = 3.3$  Hz, H-13'b]. These signals are typical of exomethylene protons conjugated with the lactonic carbonyl of sesquiterpene lactones. This was supported by the HMQC spectrum, where the H-13a and H-13b protons correlated with C-7 ( $\delta_{\text{C}} = 43.5$ ), C-11 ( $\delta_{\text{C}} = 138.7$ ) and C-12 ( $\delta_{\text{C}} = 170.7$ ). The H-13'a and H-13'b protons correlated with C-7' ( $\delta_{\text{C}} = 46.9$ ), C-11' ( $\delta_{\text{C}} = 138.2$ ) and C-12' ( $\delta_{\text{C}} = 169.5$ ). Finally, the H-6 and H-6' proton resonances were at  $\delta = 4.21$  dd and  $\delta = 4.06$  dd were confirmed by their correlation in the HSQC spectra to carbon resonances at  $\delta_{\text{C}} = 84.2$  and  $83.2$  respectively. The COSY spectrum showed the correlations of H-13a with H-13b and H13'a with H-13'b.

The lack of signals for H-1' and H-2' and the presence of the quaternary carbons corresponding to  $\delta = 158.5$  (C-1') and  $\delta = 144.8$  (C-2') indicated a tetrasubstituted  $\text{C}_1 - \text{C}_2$  double bond. The other double bond at C-9' - C-10' was determined from the HSQC spectrum, C-9' ( $\delta_{\text{C}} = 130.9$ ) and C-10' ( $\delta_{\text{C}} = 134.4$ ) as well as the proton shift at  $\delta = 5.98$  [d,  $J = 6.6$  Hz, H-9']. The HMQC showed correlations of H-9' with C-1', C-7' and C-15'. The presence of the double bond exomethylene protons (H-14'a and H-14'b) was confirmed by the absence of the proton signals at C-4' ( $\delta_{\text{C}} = 141.2$ ) as shown in the HSQC spectrum and the HMQC correlations with C-3', C-4' and C-5'. The other double bond exomethylene protons (H-14a and H-14b) was situated at C-10 ( $\delta_{\text{C}} = 150.7$ ) as confirmed by the HSQC. The carbonyl resonances at C-3 ( $\delta_{\text{C}} = 220.2$ ) and C-3' ( $\delta_{\text{C}} = 193.1$ ) were confirmed in the HMQC spectra by correlations from H-1, H-2 $\alpha$ , H-2 $\beta$ , H-5, H-15 $\alpha$  and H-14'a, H-14'b, respectively.

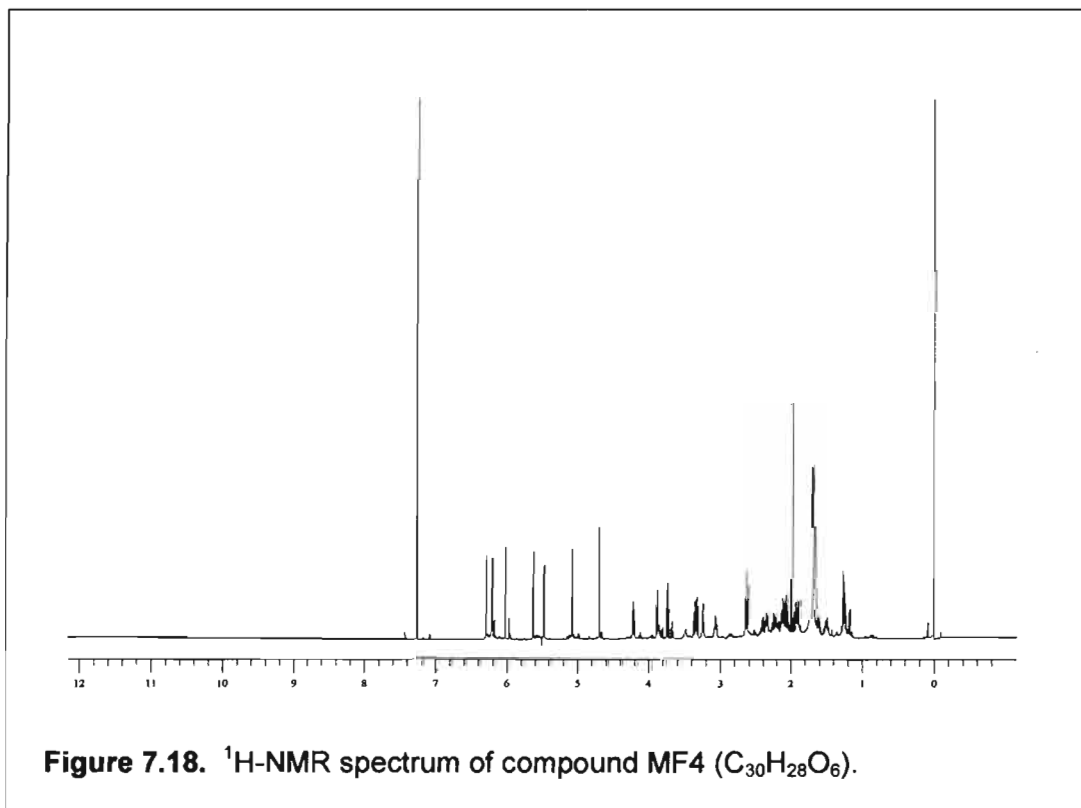
Compound MF7A was established as an unsymmetrical dimeric sesquiterpene with an unusual six-membered ring linking the two monomers. The six-membered ring was deduced from the HMQC spectrum where H-15 ( $\delta_C = 31.9$ ) correlated with C-3, C-4, C-5 and C-15' and H-5 ( $\delta_C = 50.1$ ) correlated with C-2' C-4, C-6 and C-15 and H-9' ( $\delta_C = 130.9$ ) correlated with C-1', C-7' and C-15'. Both monomers contained five-membered rings fused to seven-membered ring and the lactone rings were attached at carbons 6 and 7.

Examples of the chemical structures of dimeric guaianolides with different links between the two monomers [Trendafilova et al., 2006; Hou et al., 2003; Wong & Brown, 2002] are shown in Appendix F.

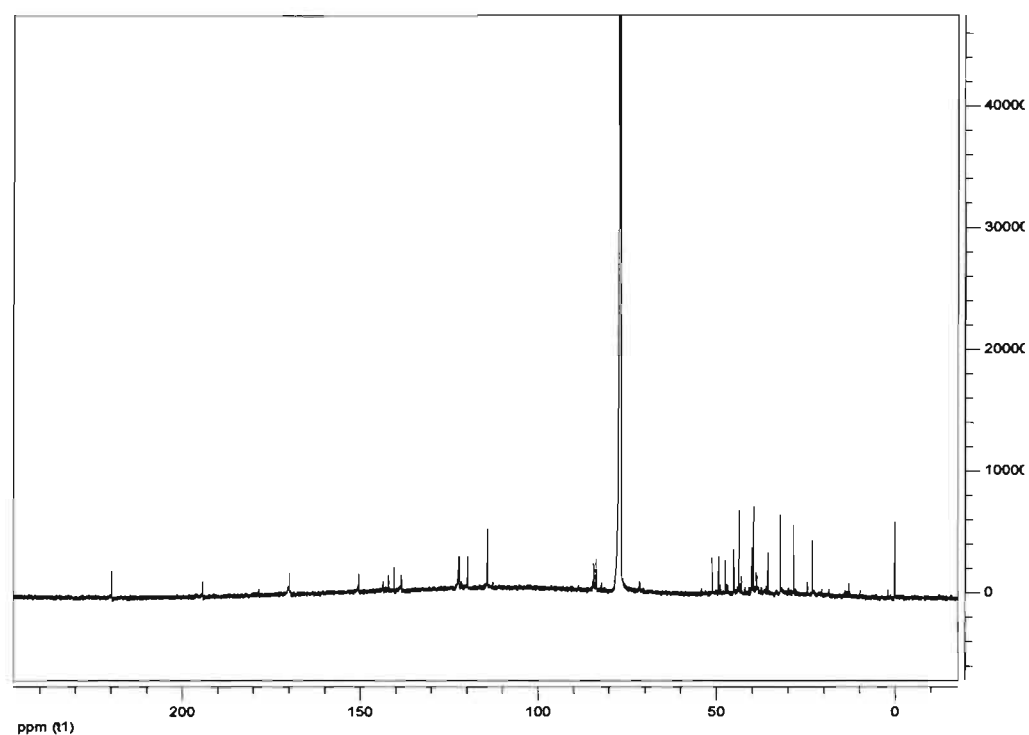
The chemical structure of compound MF7A (figure 7.17) has shown that this compound is a novel dimeric sesquiterpene lactone of the guaianolide-type. Compound MF7A has been included in a list of compounds that are in the process of being patented. The patent is held by the University of Cape Town and the Medical Research Council of South Africa.

### 7.3.5.2. NMR spectra of Compound MF4

#### 7.3.5.2.1. $^1\text{H}$ -NMR spectrum

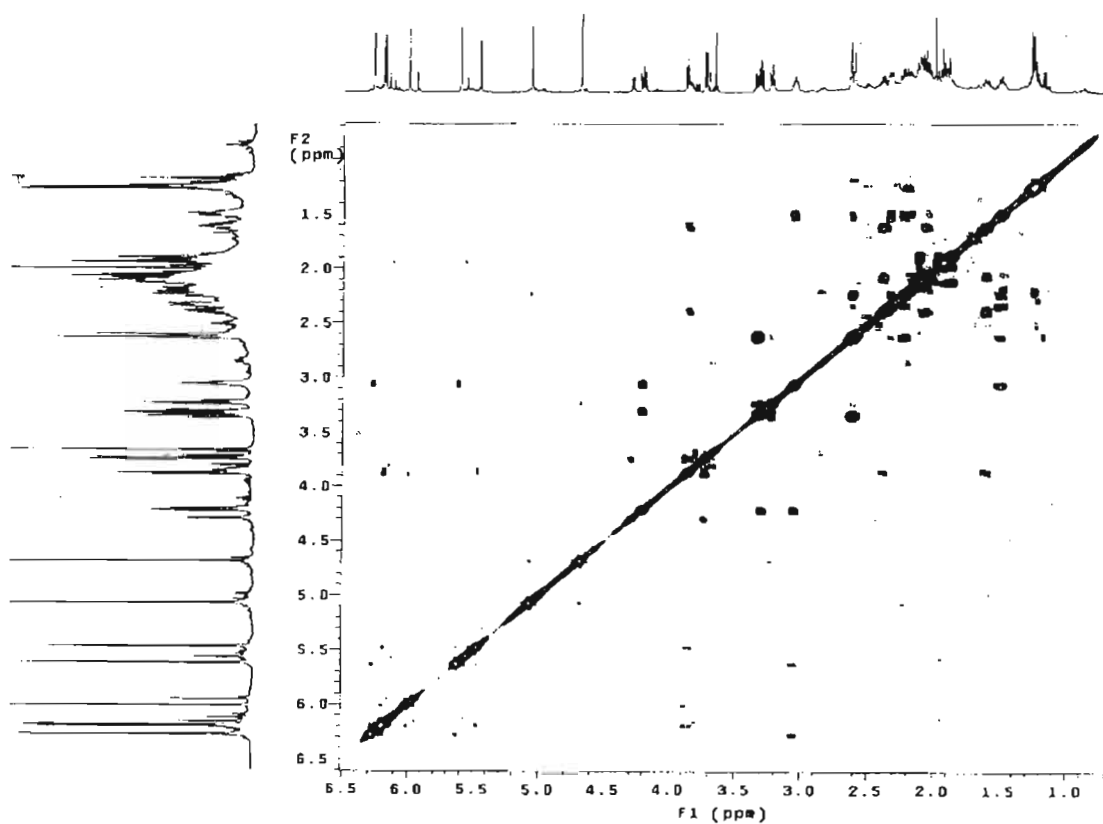


**Figure 7.18.**  $^1\text{H}$ -NMR spectrum of compound MF4 ( $\text{C}_{30}\text{H}_{28}\text{O}_6$ ).

**7.3.5.2.2.  $^{13}\text{C}$ -NMR spectrum**

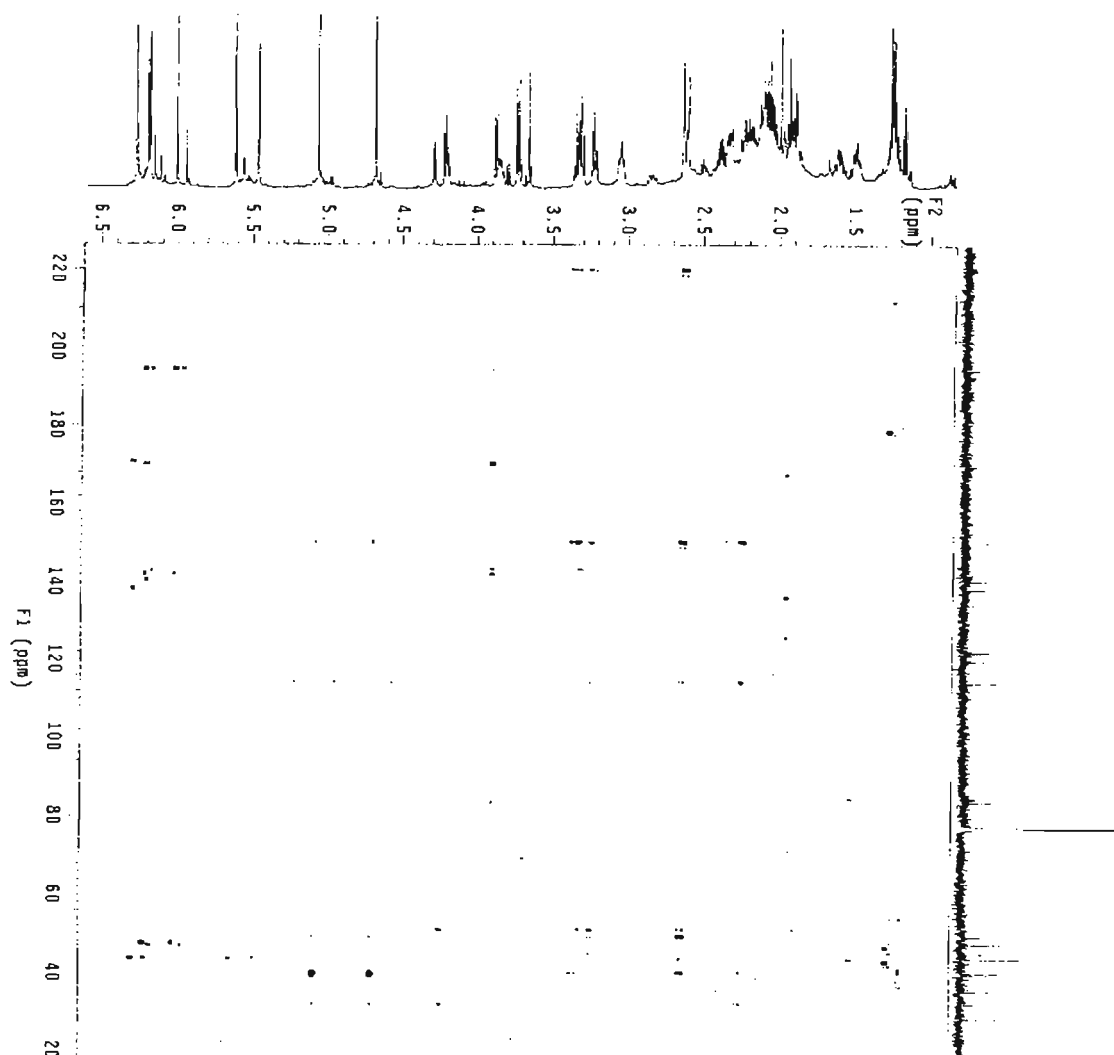
**Figure 7.19.**  $^{13}\text{C}$ -NMR spectrum of compound MF4 ( $\text{C}_{30}\text{H}_{28}\text{O}_6$ ).

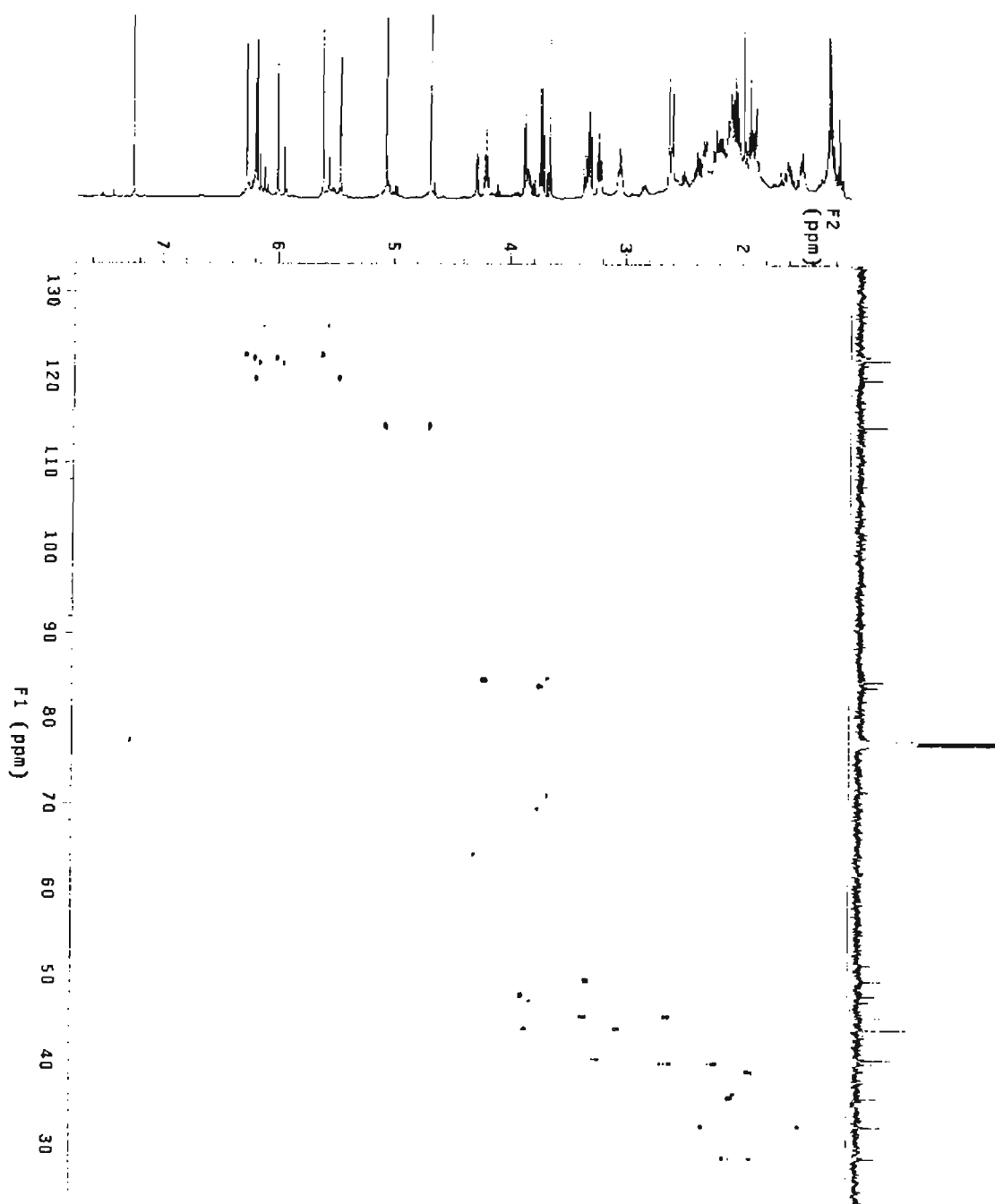
### 7.3.5.2.3. Correlation Spectroscopy (COSY)



**Figure 7.20.** COSY spectrum of compound MF4 (C<sub>30</sub>H<sub>28</sub>O<sub>6</sub>).

## 7.3.5.2.4. 2D-NMR spectrum (HMQC and HSQC)

**Figure 7.21.** HMQC spectrum of compound MF4 (C<sub>30</sub>H<sub>28</sub>O<sub>6</sub>).



**Figure 7.22.** HSQC spectrum of compound MF4 (C<sub>30</sub>H<sub>28</sub>O<sub>6</sub>).



Although compound MF4 had the same molecular mass as compound MF7A (485.1964), there was difficulty in elucidating its chemical structure. It can therefore be inconclusively said that compound MF4 is also a dimeric sesquiterpene since there was a strong indication that it is an isomer of compound MF7A. The two compounds were screened for *in vitro* antiplasmodial activity against three strains of *P. falciparum*.

### 7.3.6. Antiplasmodial Activity and Cytotoxicity of Compounds MF4 and MF7A

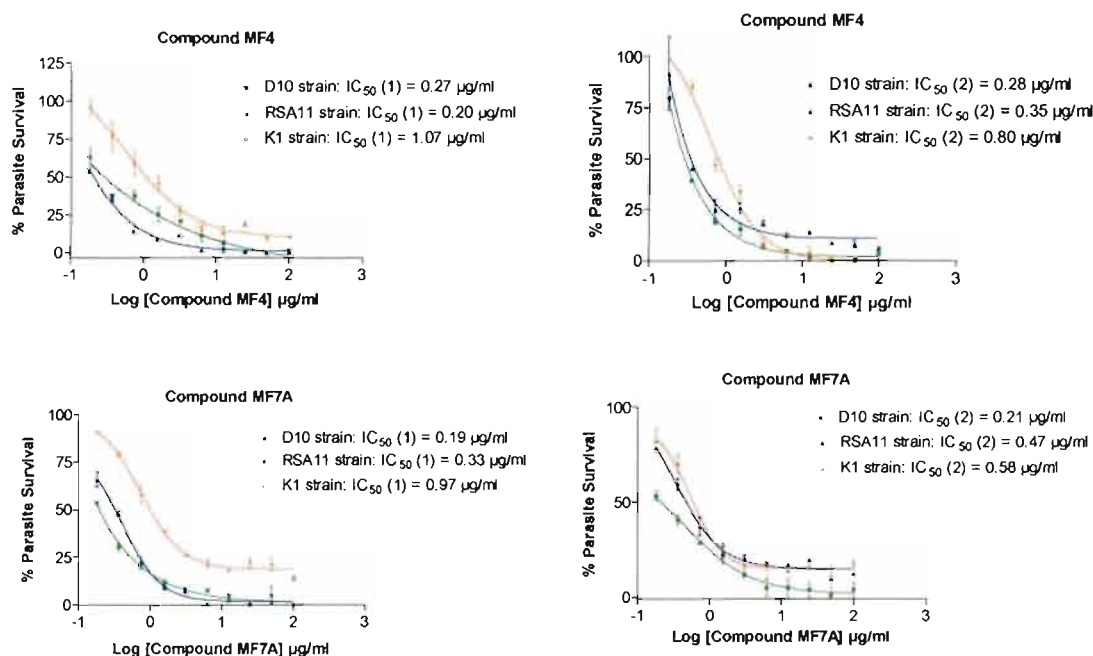
**Table 7.2.** The *in vitro* antiplasmodial activity of compounds MF4 and MF7A on CQS (D10) and CQR (K1 and RSA11) strains of *P. falciparum* and their cytotoxicity on CHO cells.

Test sample	D10 strain IC <sub>50</sub> ± S.D. (µg/ml)	K1 strain IC <sub>50</sub> ± S.D. (µg/ml)	RSA11 strain IC <sub>50</sub> ± S.D. (µg/ml)	CHO cells IC <sub>50</sub> ± S.D. (µg/ml)	SI <sup>a</sup>	RI <sup>b</sup>
MF4	0.28 ± 0.01	0.94 ± 0.14	0.28 ± 0.075	4.41 ± 0.47	15.75	3.36
MF7A	0.20 ± 0.01	0.78 ± 0.2	0.40 ± 0.07	0.45 ± 0.01	2.25	3.90
MeCN	> 100	> 100	> 100	> 100		
CQ <sup>c</sup>	11.68 ± 1.90	165.44 ± 4.38	197.68 ± 5.81	> 100	>100	14.16
Emetine	ND	ND	ND	0.053 ± 0.0045		

<sup>a</sup>SI = Selectivity index (SI) = CHO IC<sub>50</sub>/D10 IC<sub>50</sub>

<sup>b</sup>RI = Resistance index (RI) = K1 IC<sub>50</sub>/D10 IC<sub>50</sub>

<sup>c</sup>CQ = Chloroquine IC<sub>50</sub> values in ng/ml



**Figure 7.23.** The dose-response curves of the antiplasmodial activity of MF4 and MF7A on D10, K1 and RSA11 strains of *P. falciparum*. Each point is the mean of 2 tests and the error bars are S.D. of the mean.

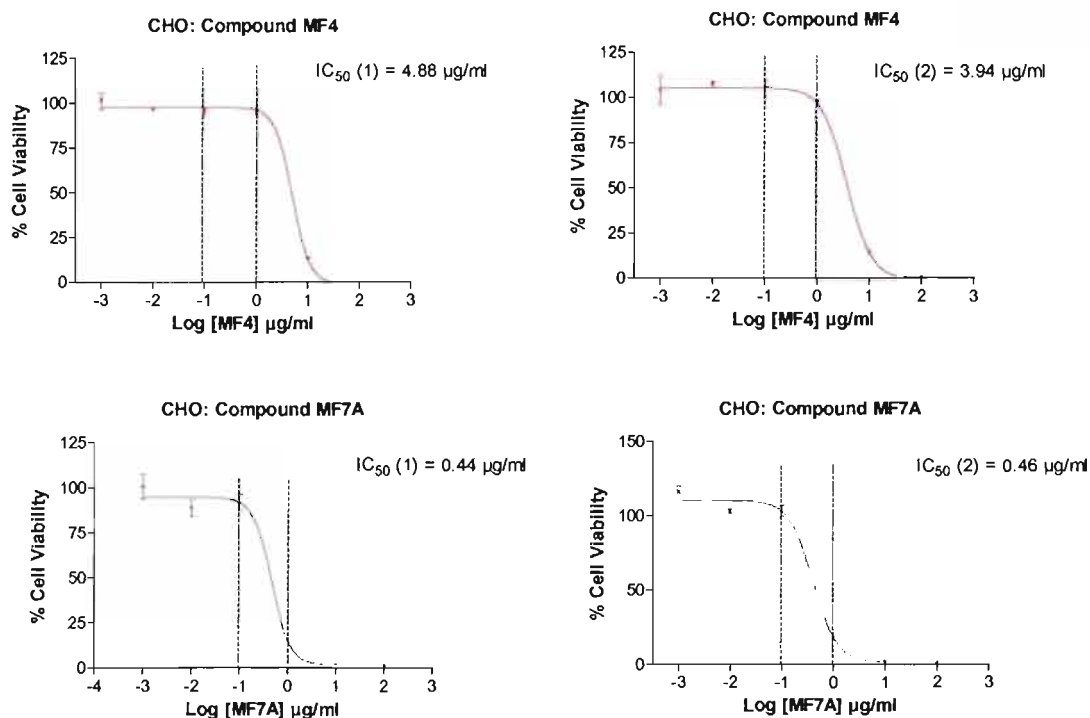
Results of the antiplasmodial and cytotoxicity screening are summarized in table 7.2 and figures 7.23, 7.24 and 7.25. Compounds MF4 and MF7A showed the highest antiplasmodial activity against the CQS strain, D10 ( $0.28 \pm 0.01 \mu\text{g/ml}$  and  $0.20 \pm 0.01 \mu\text{g/ml}$ , respectively) with compound MF7A being slightly more active than compound MF4. The two compounds also showed activity against the CQR strains, K1 and RSA11, and were more active on the RSA11 strain (MF4:  $0.28 \pm 0.075 \mu\text{g/ml}$ ; MF7A:  $0.40 \pm 0.07 \mu\text{g/ml}$ ) than on the K1 strain (MF4:  $0.94 \pm 0.14 \mu\text{g/ml}$ ; MF7A:  $0.78 \pm 0.20 \mu\text{g/ml}$ ).

The antiplasmodial activity of compound MF4 was the same against D10 and RSA11 strains and lower against the K1 strain. This indicated the possibility that

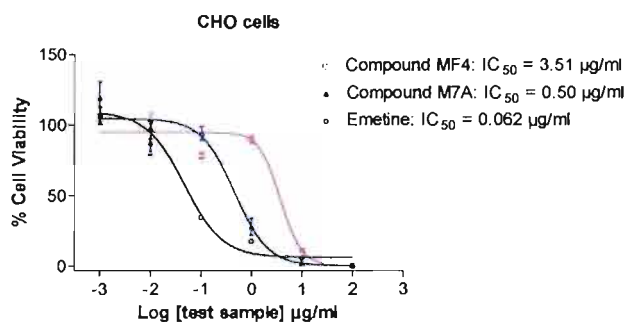
the RSA11 strain does not have the mutant gene that would confer resistance to compound MF4. Compound MF7A was 2 times more active on the D10 strain than on the RSA11 strain and as with compound MF4, the activity against the K1 strain was lower. Compounds MF4 and MF7A showed resistance indexes of 3.21 and 3.90, respectively, indicating that compounds MF4 and MF7A could have partial resistance to the parasites. The compounds did not show cross-resistance with chloroquine (RI = 14.16).

Although the two compounds possessed remarkable antiplasmodial activity, they exhibited higher cytotoxicity (MF4:  $4.41 \pm 0.47$   $\mu\text{g/ml}$ , MF7A:  $0.45 \pm 0.01$   $\mu\text{g/ml}$ ). However, the antiplasmodial activity was not due to the toxicity except for the activity of compound MF7A against K1 strain. Compound MF4 was the most favourable with the selectivity index of approximately 16 times more potent to the parasites than to the cells while compound MF7A was only 2 times more potent to the parasites than to the cells, showing lack of selectivity.

The cytotoxicity of the two compounds was very high despite the fact that the results were averages of two independent experiments. The  $\text{IC}_{50}$  of the positive control, emetine ( $0.053 \pm 0.0045$   $\mu\text{g/ml}$ ) indicated that the CHO cells were in a good state and therefore, the experiments worked well. The solvent control, acetonitrile, did not have any effect ( $\text{IC}_{50} = > 100$   $\mu\text{g/ml}$ ) on the antiplasmodial activity and the cytotoxicity of compounds MF4 and MF7A (figure 7.25). To confirm the cytotoxicity results of compounds MF4 and MF7A on CHO cells, the compounds were collected from newly prepared samples of fractions 40 and 50 as previously described. After purification, the two compounds were screened for cytotoxicity on a new batch of CHO cells and the results obtained were still similar (figure 7.25).



**Figure 7.24.** The dose-response curves of the cytotoxicity of MF4 and MF7A on CHO cells. Each point is the mean of 3 tests and the error bars are SD of the mean. The areas marked with demarcated lines represent the area where the antiplasmodial activity of the compounds on the D10 strain of *P. falciparum* was.



**Figure 7.25.** The cytotoxicity dose response curves of emetine, MF4 and MF7A. MF4 and MF7A were isolated from a new batch of fraction 40 and screened on a new batch of CHO cells, to confirm the cytotoxicity of the two compounds. Each point is the mean of 3 tests and the error bars are S.D. of the mean.

Compounds MF4 and MF7A had similar antiplasmodial activity ( $0.28 \pm 0.01$   $\mu\text{g/ml}$  and  $0.22 \pm 0.03$   $\mu\text{g/ml}$ , respectively) against the CQ-sensitive strain, D10. (table 7.3, figures 7.23). Compound MF7A was slightly more active than compound MF4. Although the two compounds were cytotoxic (MF4:  $4.41 \pm 0.47$   $\mu\text{g/ml}$  and MF7A:  $0.45 \pm 0.01$   $\mu\text{g/ml}$ )(table 7.3 and figures 7.24 and 7.25), their antiplasmodial activity was not due to the toxicity as indicated by their selectivity indexes (SI). The selectivity index of MF4 showed that it was about 16 times more potent to the parasites than to the cells while that of compound MF7A was 2 indicating lack of selectivity.

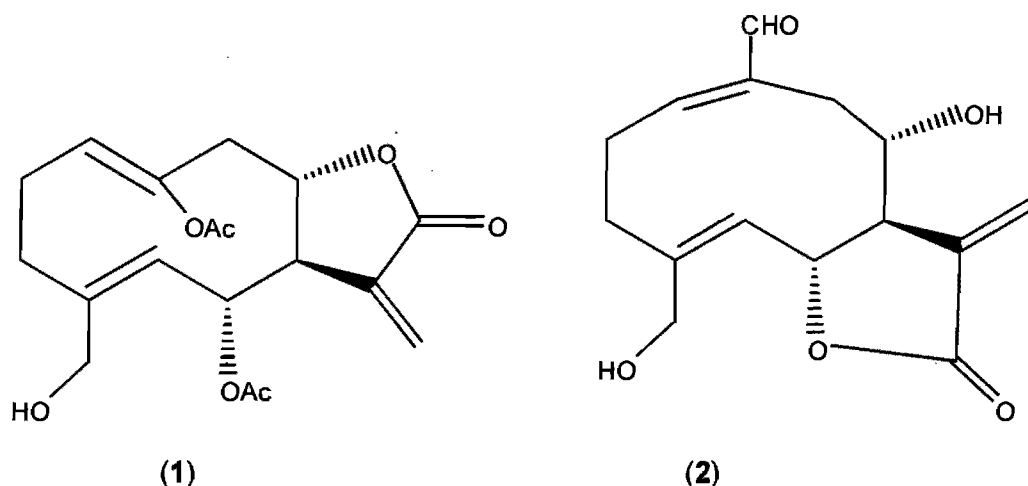
The remarkable antiplasmodial activity of compounds MF4 and MF7A has shown that they have potential as lead compounds, but the low selectivity index of compound MF4 and the lack of selectivity of compound MF7A indicated that these compounds have a narrow safety margin, which may hamper their potential as lead compounds. However, although the two compounds showed similar antiplasmodial activity against the D10 strain, there was a great difference in their cytotoxicity. Compound MF7A was about 10 times more cytotoxic on CHO cells than MF4. Therefore, based on these observations, it is possible that structural changes in both compounds could lead to derivatives that are highly active against the malaria parasites but less cytotoxic on the mammalian cells.

The antiplasmodial activity of the compounds was higher than that of the crude DCM root extract ( $2.48 \pm 0.005$   $\mu\text{g/ml} \leq \text{IC}_{50} \leq 1.55 \pm 0.07$   $\mu\text{g/ml}$ ), fraction 40 ( $0.72 \pm 0.20$   $\mu\text{g/ml} \leq \text{IC}_{50} \leq 0.63 \pm 0.045$   $\mu\text{g/ml}$ ) and fraction 50 ( $0.63 \pm 0.005$   $\mu\text{g/ml} \leq \text{IC}_{50} \leq 0.54 \pm 0.11$   $\mu\text{g/ml}$ ). The activity was therefore not lost during the fractionation and the isolation of compounds MF4 and MF7A.

### **7.3.7. Compounds isolated from *Dicoma anomala***

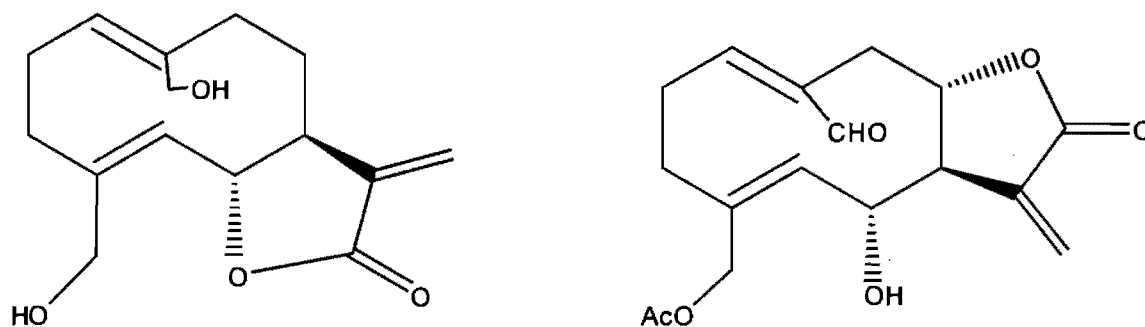
Compounds isolated from *Dicoma anomala* Sond. include sesquiterpene lactones, lupeol, lupeol acetate, phytol, germacrane D and were isolated from the aerial parts [Zdero and Bohlmann, 1990; Bohlmann et al., 1982 and Bohlmann

and le Van, 1978]. Some of the sesquiterpene lactones isolated from *D. anomala* are shown below in figure 7.26.

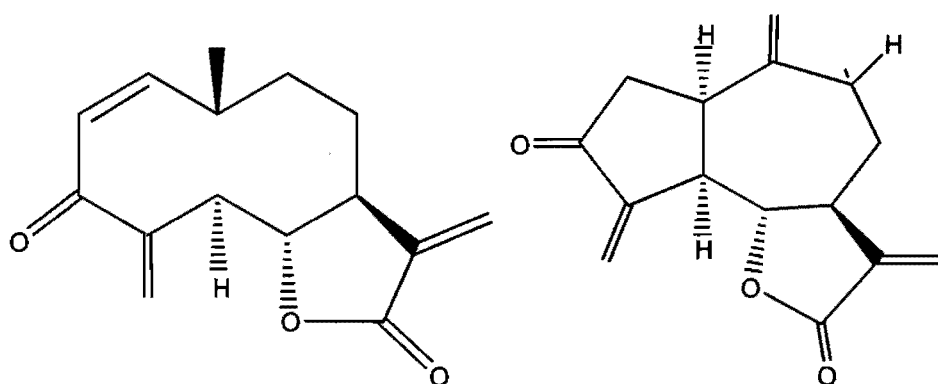


**Figure 7.26.** Sesquiterpene lactones isolated from the aerial parts of *D. anomala* Sond. ssp. *anomala* [Bohlmann, 1982 (1); Zdero, 1990 (2)].

Although Bohlmann et al., isolated sesquiterpene lactones and other compounds from the roots of *D. anomala* subsp. *cirsiioides* (figures 7.27 and 7.28), they reported that the roots of *D. anomala* subsp. *anomala* gave no characteristic compounds. But, the findings by Dr. Matsabisa [PhD thesis, University Of Cape Town, 2001] have shown for the first time that the roots of *D. anomala* subsp. *anomala* contain sesquiterpene lactones, which had molecular formulae of  $C_{30}H_{36}O_7$  ( $[M + 1]^+ = 509.2545$ ) and  $C_{30}H_{34}O_7$  ( $[M + 1]^+ = 507.2388$ ). The presence of 30 carbon atoms suggested that the structures might be dimeric sesquiterpenes. The two compounds were isolated as a mixture and their structural elucidation was incomplete due to the difficulty in separating them. The problem of separating compounds and obtaining their structures was also experienced with the isolation of compounds MF4 and MF7A as discussed in section 7.3.2 of this chapter and in Chapter 4, section 4.4, p. 70-71).



**Figure 7.27.** Sesquiterpene lactones isolated from the aerial parts of *D. anomala* Sond. ssp. *cirsioides* (Harv) Willd [Bohlmann, 1978].



**Figure 7.28.** Sesquiterpene lactones isolated from the roots of *D. anomala* Sond. ssp. *cirsioides* (Harv) Willd [Bohlmann, 1978].

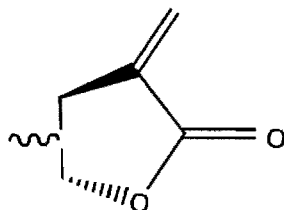
Very little scientific work has been done on *D. anomala*. The work by Zdero and Bohlmann on *D. anomala*, reported on the isolation of the compounds but not the biological activity. Matsabisa has shown in his PhD thesis that a mixture of two compounds from the roots of *D. anomala* possessed antiplasmodial activity.

The presence of dimeric sesquiterpene lactones in the roots of *D. anomala* Sond. has been confirmed in this thesis with the isolation of compounds MF4 (C<sub>30</sub>H<sub>28</sub>O<sub>6</sub>) and MF7A (C<sub>30</sub>H<sub>28</sub>O<sub>6</sub>) and the structural elucidation of compound MF7A (figure 7.17).

### 7.3.8. The activities of sesquiterpene lactones

Sesquiterpene lactones have been shown to exhibit a wide range of biological activities such as trypanocidal [Schinor et al., 2004], anticancer [Ohno et al., 2005; Cho et al., 2004; Jang et al., 1999; Sun et al., 2003], antitumour [Beekman et al., 1998], antifungal [Skaltsa et al., 2000; Vajs et al., 1999; Inoue et al., 1995], antimycobacterial [Cantrell et al., 1998; Fischer et al., 1998], antibacterial [Rabe et al., 2002; Taylor & Towers, 1998] and inflammatory activity [Lindenmeyer et al., 2006; Siedle et al., 2004; Matsuda et al., 2000; Rüngeler et al., 1999 and Lyß et al., 1998]. Pharmacological effects include inhibitory effects on alcohol absorption [Yoshikawa et al., 2000] and relaxing properties on smooth muscles [Campos et al., 2003]. Sesquiterpene lactones have also been shown to possess herbicidal and insecticidal activity effects [Guillet et al., Collins et al., 2000; Macías et al., 1999, 2000].

Some of the activities of sesquiterpene lactones have been shown to be due to the presence of the  $\alpha$ -methylene- $\gamma$ -lactone group shown in figure 7.29.



**Figure 7.29.** The  $\alpha$ -methylene- $\gamma$ -lactone group.

Lee et al., and Matsuda et al., have shown that the  $\alpha$ -methylene- $\gamma$ -lactone moiety was important for the inhibitory effects of sesquiterpene lactones on NO production. Compounds lacking this moiety showed weaker inhibitory activities. Reduction with the opening of the lactone ring also showed weaker inhibitory activity on NO production [Matsuda et al., 2000]. The reductive opening of the lactone ring resulted in no activity against *Mycobacterium tuberculosis* [Fisher et



al., 1998] and against the inhibition of alcohol absorption [Yoshikawa et al., 2000].

The  $\alpha$ -methylene- $\gamma$ -lactone group is essential but not sufficient for the biological activity of sesquiterpene lactones. The activity of sesquiterpene lactones containing the  $\alpha$ -methylene- $\gamma$ -lactone group may be enhanced by the presence of epoxides, conjugated carbonyl, keto and aldehyde groups [Saroglou et al., 2005; Siedle et al., 2004; Fischer et al., 1998]. On the other hand, the presence and the increasing number of hydroxyl groups have been shown to diminish or reduce the inhibitory activity of sesquiterpene lactones on the transcription factor NF- $\kappa$ B [Siedle et al., 2004], the cytotoxicity on cancer cell lines [Sun et al., 2003] and the antimycobacterial activity [Cantrell et al., 1998]. The hydroxyl groups increase the polarity of the compounds, which reduces their lipophilicity and therefore their penetration through the highly lipophilic cell walls and plasma membranes.

However, the  $\alpha$ -methylene- $\gamma$ -lactone group is not a prerequisite for activity. This has been shown by sesquiterpenes lacking the  $\alpha$ -methylene- $\gamma$ -lactone group such as the antimalarial drug, artemisinin [Meshnick et al., 1996], sesquiterpenes that inhibited the release of elastase from granulocytes [Siedle et al., 2003] as well as those that had relaxant effects on smooth muscle [Campos et al., 2003].

## 7.4. CONCLUSION

- Two compounds, MF4 and MF7A have been isolated from *Dicoma anomala* subsp. *anomala* (Sond.) and were found to be dimeric sesquiterpenes. This was confirmed by their calculated mass  $[M + H]^+$  of 485.1964 consistent with the molecular formula of  $C_{30}H_{28}O_6$ . The chemical structure of compound MF7A confirmed that this compound was a dimeric sesquiterpene of the guainolide-type. The same molecular mass, ion mass fragmentation patterns and molecular formulae of compound MF4 and MF7A strongly indicated that these compounds are isomers, meaning that

compound MF4 has the same chemical structure as compound MF7A. There was difficulty in elucidating the chemical structure of compound MF4, this suggested that compounds MF4 and MF7A have different structural conformations.

- Compound MF4 was more potent on the CQS (D10) strain ( $0.28 \pm 0.01$   $\mu\text{g/ml}$ ) than on the CQR (K1) strain ( $0.94 \pm 0.34$   $\mu\text{g/ml}$ ) indicating partial resistance of the parasites to compound MF4. The activity was the same for CQS (D10) strain as for CQR (RSA11) strain ( $0.28$   $\mu\text{g/ml}$ ). The antiplasmodial activity of compound MF4 was 16 times more potent than cytotoxic, showing the selectivity of the compound.
- Compound MF7A also showed high potency on the CQS (D10) strain ( $0.20 \pm 0.01$   $\mu\text{g/ml}$ ) than on the CQR strains (K1) ( $0.78 \pm 0.20$   $\mu\text{g/ml}$ ) and (RSA11) ( $0.40 \pm 0.07$   $\mu\text{g/ml}$ ) indicating partial resistance of the parasites to compound MF7A. Compound MF7A showed lack of selectivity as it was only 2 times more potent to the parasites than to the cells.
- The antiplasmodial activity of compound MF4 was about 2 times more than that of fractions 40 and 50 and 6-8 times more active than that of the cold DCM root extract. The activity of compound MF7A was about 3 times more active than that of fractions 40 and 50 and 8-12 times more active than of the cold DCM root extract. No loss of activity, therefore, resulted from the fractionation of the cold DCM extract.
- The results obtained have shown that *D. anomala* (Sond.) not only relieve malaria symptoms such as headache and fever but has the biological activity against the malaria parasite, *P. falciparum*.
- The two compounds, MF4 and MF7A, showed promising antimalarial activity but have narrow safety margin. The chemical structure of

compound MF4 still need to be elucidated and derivatives of both compounds prepared to determine whether they would be less cytotoxic with high antiplasmodial activity. These compounds may thus be potentially useful as lead compounds for the development of a new class of antimalarial drugs.

- The MS of peaks MF7B and MF7C and their UV spectra indicated that they have similar chemical structures to compound MF7A and are therefore dimeric sesquiterpenes.
- The MS of peaks MF5, MF6B, MF6E and MF8, which were present in fraction 40, have shown that the roots of *D. anomala* (Sond.) contain compounds with similar chemical structures and which are possible dimeric sesquiterpenes.

# ***CHAPTER 8***

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## **DISCUSSION AND CONCLUSIONS**

**Objectives achieved, Problems encountered and Future work**

## 8.1. OBJECTIVES ACHIEVED

The objectives of this project were:

- To screen the leaf and root extracts of *Dicoma anomala* for antiplasmodial activity against the CQS strain of *P. falciparum*.
- To isolate compounds from *Dicoma anomala* and screen them for antiplasmodial activity against the CQS and CQR strains of *P. falciparum*.
- To determine the chemical structures of the isolated compounds using spectroscopic techniques such as mass spectrometry and NMR.
- To determine the cytotoxicity of the antiplasmodially active extracts and compounds on CHO cells.

All of the objectives were achieved. The plant material was collected from four locations (Lesotho, Sterkspruit, Reitz and Limpopo). Cold and hot extracts of the leaves and twigs (a mixture) and the roots were prepared for the antiplasmodial screening. Any test sample that showed antiplasmodial activity  $\leq 10 \mu\text{g/ml}$  was considered active.

The cold DCM leaf extracts of the plant collected in Sterkspruit, Reitz and Limpopo were active against the CQS (D10) strain of *P. falciparum* ( $0.68 \pm 0.025 \mu\text{g/ml} \leq \text{IC}_{50} \leq 3.14 \pm 0.12 \mu\text{g/ml}$ ). The cold and hot DCM root extracts from the four locations also showed activity against the malarial parasites ( $\text{IC}_{50} \leq 2.48 \mu\text{g/ml}$ ). Although the selectivity indexes of the extracts were low (ranging from 2 – 9), they showed that the antiplasmodial activity of the leaf and root extracts were selective for the malarial parasites and therefore the activity was not due to cytotoxicity.

The cold H<sub>2</sub>O root extracts ( $13.92 \pm 0.52 \mu\text{g/ml} \leq \text{IC}_{50} \leq 29.76 \pm 2.90 \mu\text{g/ml}$ ), the hot H<sub>2</sub>O root extracts prepared on the soxhlet for 6 hrs ( $\text{IC}_{50} \geq 61.15 \mu\text{g/ml}$ ) and the hot H<sub>2</sub>O root extracts prepared on the hotplate for 6 hrs ( $17.06 \pm 1.22 \mu\text{g/ml} \leq \text{IC}_{50} \leq 69.46 \pm 2.16 \mu\text{g/ml}$ ) did not show significant antiplasmodial activity. The

hot H<sub>2</sub>O root extracts from Lesotho and Sterkspruit, prepared on the hotplate for 2 hrs also did not show significant activity ( $24.16 \pm 0.16$  µg/ml and  $19.54 \pm 0.045$  µg/ml, respectively) while the hot H<sub>2</sub>O root extract of Limpopo ( $IC_{50} = 8.44 \pm 0.25$  µg/ml) showed moderate activity. The activity could have been influenced by the short time exposure to heat (2 hrs as opposed to 6 hrs), the presence and the concentration of antiplasmodial compounds in the hot H<sub>2</sub>O root extract. The extract showed less cytotoxicity ( $87.06 \pm 6.70$  µg/ml) on CHO cells.

The plant roots from Lesotho were also extracted with other organic solvents. The chloroform and ethylacetate root extracts showed good activity ( $1.18 \pm 0.08$  µg/ml and  $1.56 \pm 0.36$  µg/ml, respectively), which was similar to that of the cold DCM extract from batch II ( $1.55 \pm 0.07$  µg/ml). The similar antiplasmodial activity was also supported by the similarity in the HPLC chromatograms of the three extracts. The selectivity indexes (4 - 10) indicated that the extracts were selective towards the malarial parasites and that their activity was not due to cytotoxicity. The hexane, petroleum spirit and methanol extracts of the roots did not exhibit any significant antiplasmodial activity ( $10.67 \pm 0.32$  µg/ml  $\leq IC_{50} \leq 39.30 \pm 0.70$  µg/ml).

Four fractions were obtained from the cold H<sub>2</sub>O extraction of the roots of *D. anomala* from Lesotho. These fractions differed from the most concentrated to the less concentrated extraction. The fractions were screened for antiplasmodial activity and the activity was found in the third and the fourth fractions. These results were observed in two separate extractions. In the first extraction, with fractions denoted by H201 – H204, H204 was more active than H203 and in the second extraction, with fractions denoted by H1 – H4, H3 was more active than H4. Fractions H203, H204, H3 and H4 ( $7.48 \pm 0.07$  µg/ml,  $5.54 \pm 0.16$  µg/ml,  $4.85 \pm 0.18$  µg/ml and  $7.0 \pm 1.20$  µg/ml, respectively) were active compared to the cold H<sub>2</sub>O root extract ( $23.86 \pm 0.90$  µg/ml), which was not separated. The four fractions had less cytotoxic effects on the CHO cells (H203 =  $66.74 \pm 2.92$  µg/ml, H204 =  $60.43 \pm 2.09$  µg/ml, H3 =  $59.60 \pm 2.20$  µg/ml, H4 =  $78.73 \pm 1.99$  µg/ml)

and these results indicated that the antiplasmodial activity of the samples was not due to the cytotoxicity. The selectivity of the antiplasmodial potency was high by a magnitude of 12 for fractions H204 and H3, 11 for fraction H4 and 9 for fraction H203. The results have shown that the roots of *D. anomala* do not only treat malarial symptoms such as fever, but also have biological effects on the malarial parasites.

The cold DCM root extract from Lesotho (batch I and II) was purified using SPE. The compounds in the crude DCM extract were separated using 10% (0% - 100%) increment mobile phases of MeCN and H<sub>2</sub>O to afford 13 fractions. The most active fractions were fractions 30, 40 and 50 ( $0.46 \pm 0.12 \mu\text{g/ml} \leq \text{IC}_{50} \leq 0.72 \pm 0.20 \mu\text{g/ml}$ ) and the work was continued with fractions 40 and 50. To separate the compounds in fractions 40 and 50, the crude DCM extract was further separated with 5% (fractions 35 - 55) and 1% (fractions 31 - 35) increment mobile phases of MeCN and H<sub>2</sub>O. Fractions 35 - 55 showed activity against D10 strain ( $0.23 \pm 0.025 \mu\text{g/ml} \leq \text{IC}_{50} \leq 1.09 \pm 0.005 \mu\text{g/ml}$ ) but the fractions contained a lot of peaks. The SPE procedure for the fractionation of the cold DCM root extract (batch I and II) into 10% and 5% fractions and their antiplasmodial activity were reproducible.

The crude DCM extracts (cold and hot extracts) from Sterkspruit had similar HPLC profiles to those from Lesotho. Since fractions 30, 40 and 50 were the three most active fractions from the SPE fractionation of the cold DCM extract from Lesotho, the cold DCM extract from Sterkspruit was fractionated into fractions 30, 40 and 50 using SPE. The HPLC profiles and antiplasmodial activity of these fractions were similar to those from the crude DCM extract from Lesotho.

The HPLC profiles of fractions 31 - 35 (from batch I cold DCM root extract) showed no reduction in the number of peaks and most of the peaks that were present in 10% fractions (fractions 40 and 50) were also present in these fractions and were more enhanced. This was determined by observing their

HPLC spectra. Fractions 31 - 35 were nevertheless screened for antiplasmodial activity and showed activity against D10 strain ( $0.35 \mu\text{g/ml} \leq \text{IC}_{50} \leq 0.79 \mu\text{g/ml}$ ), K1 strain ( $0.47 \mu\text{g/ml} \leq \text{IC}_{50} \leq 0.66 \mu\text{g/ml}$ ), RSA 11 strain ( $0.25 \mu\text{g/ml} \leq \text{IC}_{50} \leq 1.12 \mu\text{g/ml}$ ) and FAC8 strain ( $0.21 \mu\text{g/ml} \leq \text{IC}_{50} \leq 8.13 \mu\text{g/ml}$ ).

Two compounds, MF4 and MF7A have been isolated from the roots of *D. anomala* from Lesotho. Compounds MF4 and MF7A were both isolated on semi-preparative HPLC from fraction 40 and only compound MF7A was isolated from fraction 50. Both compounds had a mass of  $[\text{M} + \text{H}]^+$  of 485.1964 which was consistent with the molecular formula of  $\text{C}_{30}\text{H}_{28}\text{O}_6$ . The two compounds eluted at different retention times on the semi-preparative HPLC (MF4: 11.191 minutes and MF7A: 14.047 minutes) at 60% MeCN in  $\text{H}_2\text{O}$  and flow rate of 2 ml/min. The chemical structure of compound MF7A was elucidated by NMR, MS and HRMS spectroscopy and was found to be a novel dimeric sesquiterpene of the guaianolide type. The results from the HRMS and HPLC strongly indicated that the two compounds are possible isomers. The chemical structure of compound MF4 could not be fully elucidated due to difficulty in proton and carbon assignments. Since this compound is a possible isomer of compound MF7A, it could be deduced that its chemical structure is similar to that of compound MF7A and it is also a novel dimeric sesquiterpene ( $\text{C}_{30}\text{H}_{28}\text{O}_6$ ).

Compound MF4 was highly active against the CQS strain, D10 ( $0.28 \pm 0.01 \mu\text{g/ml}$ ) and the CQR strain, RSA11 ( $0.28 \pm 0.075 \mu\text{g/ml}$ ). The activity against the two strains was similar and this indicated that compound MF4 might have a similar mode of action against D10 and RSA11 strains or the RSA11 strain had not developed resistance against this compound. Compound MF7A was also highly active against the CQS strain, D10 ( $0.20 \pm 0.01 \mu\text{g/ml}$ ) and was less active against the CQR strain, RSA11 ( $0.40 \pm 0.07 \mu\text{g/ml}$ ), which indicated that the mode of action could be different or the malarial parasites were showing resistance to this compound. Both compounds showed lower activity against the CQR strain, K1 (MF4:  $0.94 \pm 0.34 \mu\text{g/ml}$ ; MF7A:  $0.78 \pm 0.20 \mu\text{g/ml}$ ) than against



the RSA11 strain. The results showed that the two compounds could have different modes of action against the two CQR strains and the resistance indexes of the two compounds (MF4: 3.36 and MF7A: 3.90), indicated that compounds MF4 and MF7A could have partial resistance to the parasites. The compounds did not show cross-resistance with chloroquine (RI = 14.16).

Although compound MF7A showed lack of selectivity (SI = 2.25) towards the parasites, compound MF4 was the favourable one being 16 times more selective towards the parasites than the CHO cells.

Compounds MF4 and MF7A could therefore, be potential lead compounds for the development of potential antimalarial drugs since they showed antiplasmodial activity against CQS (D10) strain and CQR (K1 and RSA1) strains.

The results obtained from the crude extracts, SPE fractions, isolated compounds have shown that *Dicoma anomala* has a potential for the treatment of malaria and that there are still a lot of studies to be conducted on this plant.

There were 6 peaks which could not be isolated due to instability (peak MF5), difficulty in separation, the level of purity and quantity (peaks MF6B, MF6E, MF7B, MF7C and MF8). All these peaks were present in fraction 40. Compound MF7A and peaks MF7B and MF7C had molecular ion masses  $m/z$  at  $[M + H]^+$  which differed by two amu (485, 487 and 489, respectively) and their ion mass fragmentation patterns also differed by two amu (Chapter 7, p. 138 – 141). This indicated the hydrogenation of the double bond/s and showed that MF7B and MF7C have similar chemical structures to compound MF7A. This was further supported by the similarity in the HPLC spectra of these peaks (Chapter 7, p. 134). The ion mass fragmentation patterns showed several losses of water and possibly (alcohol, acid, glycosides).

A similar observation was made with peak MF8, which eluted after peak MF7C (Appendix E). Its molecular ion mass  $m/z$  at  $[M + H]$  was 491, indicating a difference of 2 amu from MF7C (489) and its ion mass fragmentation pattern also differed from that of MF7C by 2 amu. This means that compound MF7A, peaks MF7B, MF7C and MF8 differed from each other by 2 amu, indicating the level of hydrogenation bond/s and therefore similar chemical structures. Peaks MF5, MF6B and MF6E, which eluted after peak MF4 also had molecular ion masses which differed by 2 amu (524 for MF5 and 526 for MF6B and 528 for MF6E) (Appendix E). Their ion mass fragmentation patterns also differed by 2 amu, indicating similarity in chemical structures.

Another observation was that peaks MF5 and MF7C had common mass ions in their ion mass fragmentation patterns and similarly for peaks MF6B and MF8, and MF7B and MF8 (Appendix E).

These results have shown that the roots of *D. anomala* contain compounds with similar chemical structures and which are possible dimeric sesquiterpenes.

## 8.2. PROBLEMS ENCOUNTERED

- The isolation of compounds MF4 and MF7A was difficult due to these compounds co-eluting with other compounds, which had similar HPLC spectra to MF4 and MF7A.
- The purification of compound MF7A was more challenging than that of compound MF4. The HPLC separation between compound MF7A and MF7B was very small and this made it difficult to purify MF7A since the HPLC spectra of MF7A and MF7B were very similar. The purity of compound MF7A was therefore constantly monitored by mass spectrometry and it took months to purify so as to obtain sufficient material for biological testing and chemical studies.

- In addition to this, the yields were very low (MF4 = 1.72 mg and MF7A = 0.63 mg from 45 mg of fraction 40). The yield of the cold DCM root extract (3.65 mg) from the powdered roots (200.43 g) was also very low.

### 8.3. FUTURE WORK

1. Compounds MF4 and MF7A are dimeric sesquiterpenes and some *in vitro* studies would have to be done to determine whether upon the breaking down of the dimers into their respective monomers:

- The antiplasmodial activity would be retained or lost and if retained is it higher or lower than that of the dimers.
- The cytotoxicity would be higher or lower than that of the dimers.

2. Compounds MF4 and MF7A have shown a potential as lead compounds for the development of antimalarial drugs, but they first have to be screened for *in vivo* antimalarial activity and toxicity. This will determine if their suitable for human use. The *in vivo* screening would also address the following:

- The *in vitro* screening showed that compound MF4 was more selective to the parasites than to the cells as compared to compound MF7A but both compounds were cytotoxic on CHO cells, thereby indicating their marginal safety. It would be important to determine whether these compounds are toxic *in vivo*.
- If they are not toxic *in vivo*, then it has to be shown whether are they being metabolized to non-toxic metabolites and whether these metabolites are active antimalarials.

- The bioavailability of the compounds, which is an important factor in determining the efficacy of drugs.
3. The other important factor in the development of compounds MF4 and MF7A as potential lead antimalarials is to have compounds with less or non-cytotoxic effects. Structural modifications on compounds MF4 and MF7A could show whether their derivatives would retain or lose the antiplasmodial activity and show less or no cytotoxicity.
  4. The water fractions (H203, H204, H3 and H4) and the DCM leaf extracts from Sterkspruit, Limpopo and Reitz showed antiplasmodial activity against CQS strain (D10). The compounds in these water fractions and DCM leaf extracts have to be isolated to determine which compounds are active against *P. falciparum* parasites and how structurally different or similar are they to compounds MF4 and MF7A.
  5. Other compounds from fraction 40 and 50 should be isolated, screened for antiplasmodial activity and determine how their structures and activity compare to those of MF4 and MF7A.
  6. Peaks MF7B and MF7C (from fractions 40 and 50) and compound MF7A had similar HPLC spectra and their masses were shown to differ by 2 amu (MF7A: 485, MF7B: 487 and MF7C: 489). It would be interesting to see how the antiplasmodial activity, cytotoxicity and chemical structures of MF7B and MF7C compare to those of MF7A and MF4.
  7. Studies on the mechanism of action, including proteomics (to determine drug targets and hence possible mechanism of action), genomics (to determine possible genotoxicity), metabolimics, structure-activity and structure-cytotoxicity relationships of compound MF4 and MF7A need to be done to understand this class of compounds.

8. The success of developing compounds MF4 and MF7A as antimalarial lead compounds will depend on the availability of the plant material. The low yields of active compounds from natural sources is a major problem, which could lead to over-harvesting of the plant and in the long run could result in the extinction of the plant. The yields of compounds MF4 and MF7A obtained from 45 mg of fraction 40 were 0.38 % (0.17 mg) and 0.18% (0.08 mg), respectively. In addition to this, it will be difficult to harvest *D. anomala* since it is a small and low-lying plant which grows on rocky hillsides. The solution to this problem would be:

- To cultivate *D. anomala* on a large scale and obtain the compounds from this plant. The cultivation of the plant would involve the communities for ensuring constant supply of raw materials.
- To have an efficient synthetic approach that will result in the synthesis of compounds with desired stereochemistry. The 6,12-guaianolides [Bargues et al., 1998; 2002] and 8,12-guaianolides [Blay et al., 2000] have been synthesized through photochemical rearrangement from santonin, a commercially available natural eudesmanolide. The chemical methods could be used for the synthesis of compound MF7A (a 6,12 -guaianolide) and the monomers linked chemically to obtain the desired dimer.
- Another approach for the organic synthesis of compounds MF4 and MF7A would be the use of precursor building blocks, such as the C15 precursor (5-7-5-guaianolides).

9. The *in vivo* antimalarial activity and *in vivo* toxicity (preclinical toxicology) of the compounds isolated as well as the crude extracts and fractions that showed *in vitro* antiplasmodial activity, have to be investigated. Once promising results have been achieved and it has been shown that the compounds could be obtained on a large production scale, the pharmaceutical interest and involvement could

occur for the manufacturing and production of antimalarials from *Dicoma anomala*.

10. Peaks MF5, MF6B, MF6E, MF7B, MF7C and MF8 would have to be isolated and chemical structures obtained to determine how they differ from each other and from the chemical structure of compound MF7A. Also, to determine their antiplasmodial activity, antimalarial activity and toxicity.

11. Current research at the Medical Research Council, South Africa, is evaluating the toxicology of both the DCM and H<sub>2</sub>O root extracts on a velvet monkey model using a 90-day sub-chronic test.

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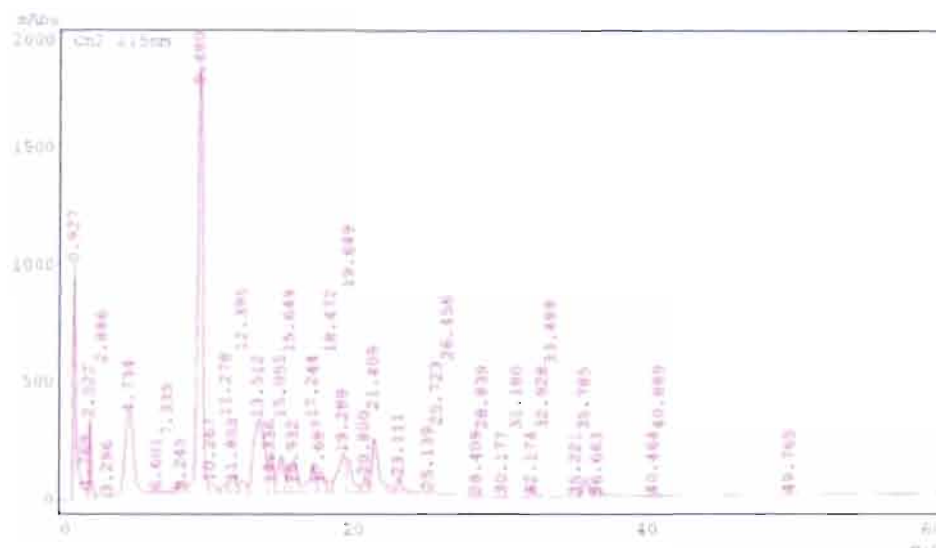
## ***LIST OF APPENDICES***

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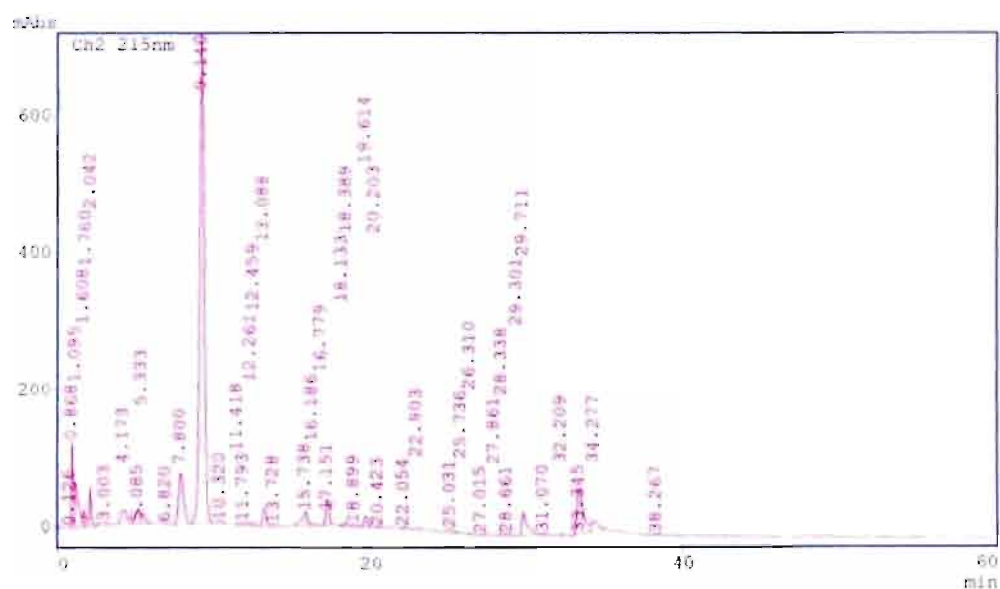
## ***APPENDIX A***

**THE HPLC CHROMATOGRAMS OF THE LEAF AND  
TWIG AND THE ROOT EXTRACTS OF *D. anomala* FROM  
STERKSPRUIT, LIMPOPO AND REITZ**







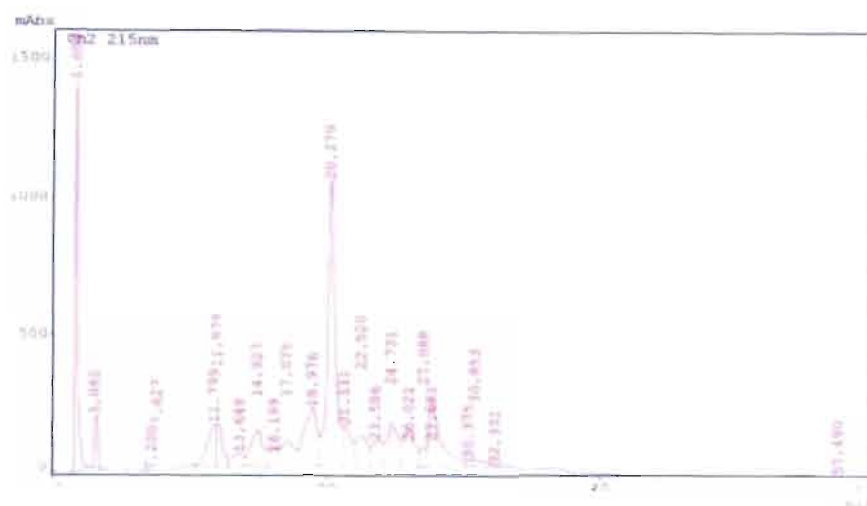


**Appendix A5.** The HPLC chromatogram of the cold DCM root extract of *D. anomala* from Reitz. The analytical separation of the extract was done on a C18 reverse phase Phenomenex column at a gradient of 20% - 100% MeCN: H<sub>2</sub>O. The flow rate was 1 ml/min, injection volume was 150 µl and the concentration was 1 mg/ml.

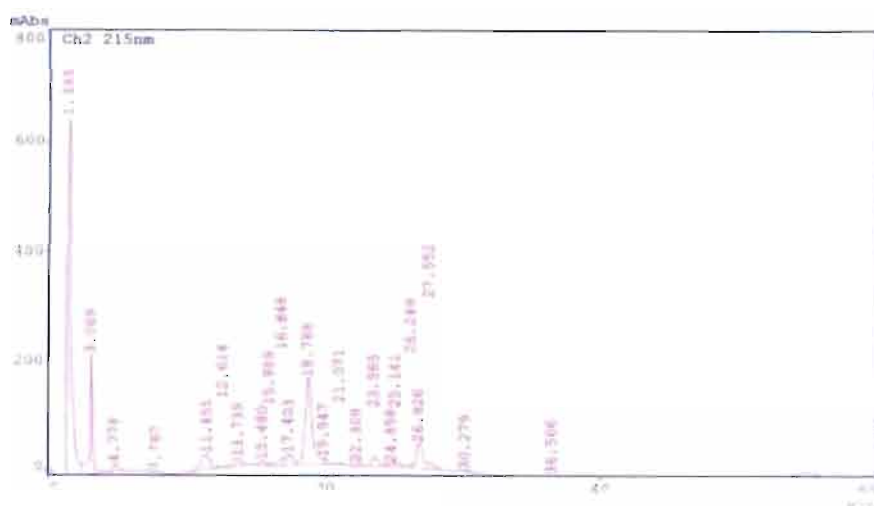
## ***APPENDIX B***



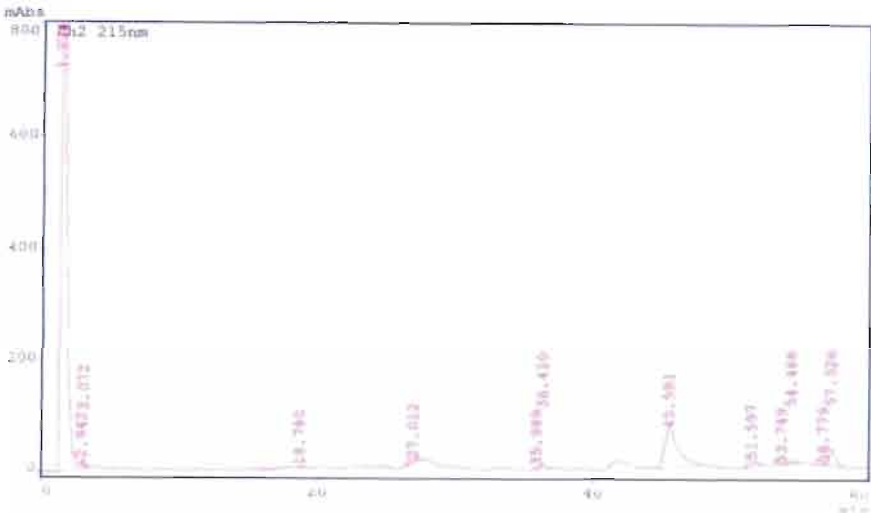
**THE HPLC CHROMATOGRAMS OF ORGANIC ROOT  
EXTRACTS OF *D. anomala* FROM LESOTHO**



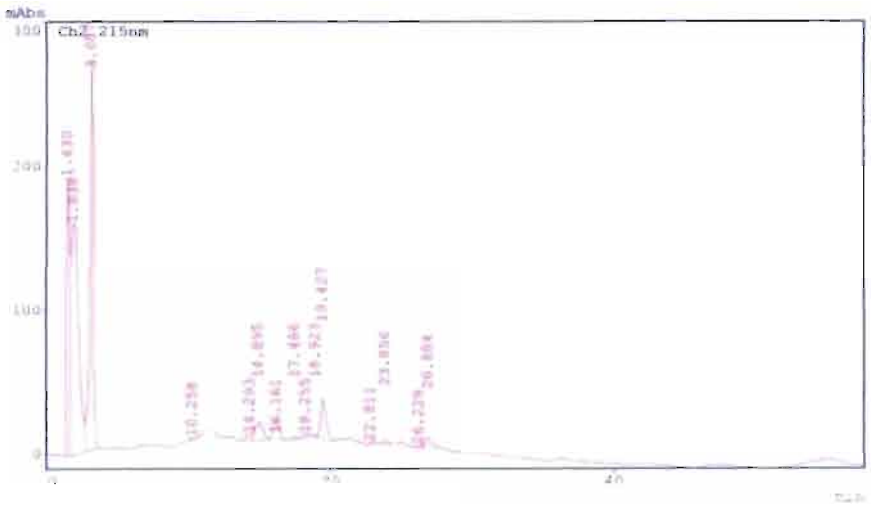
**Appendix B1.** The HPLC chromatogram of the crude chloroform root extract of *D. anomala* from Lesotho. The analytical separation of the extract was done on a C18 reverse phase Phenomenex column at a gradient of 20% - 100% MeCN: H<sub>2</sub>O. The flow rate was 1 ml/min, injection volume was 150 µl and the concentration was 1 mg/ml.



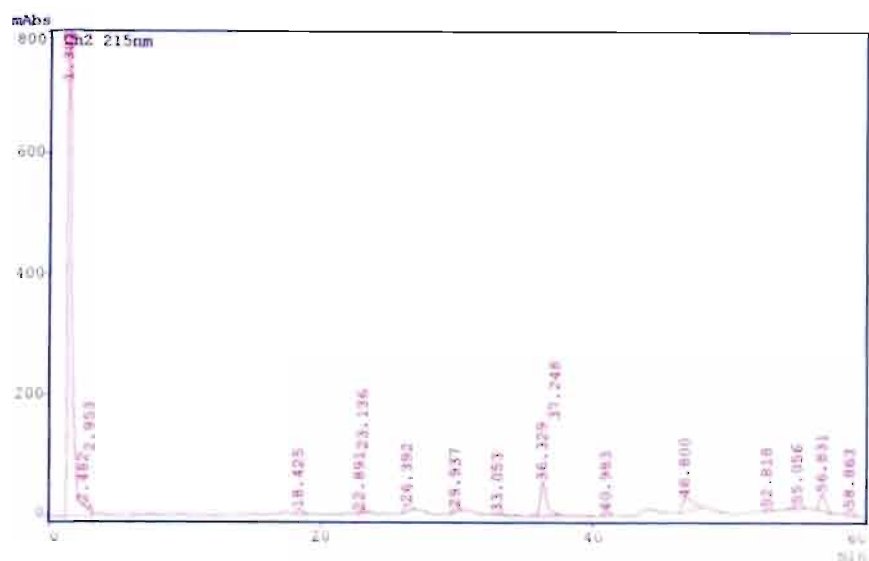
**Appendix B2.** The HPLC chromatogram of the crude ethylacetate root extract of *D. anomala* from Lesotho. The analytical separation of the extract was done on a C18 reverse phase Phenomenex column at a gradient of 20% - 100% MeCN: H<sub>2</sub>O. The flow rate was 1 ml/min, injection volume was 150 µl and the concentration was 1 mg/ml.



**Appendix B3.** The HPLC chromatogram of the crude hexaneroot extract of *D. anomala* from Lesotho. The analytical separation of the extract was done on a C18 reverse phase Phenomenex column at a gradient of 20% - 100% MeCN: H<sub>2</sub>O. The flow rate was 1 ml/min, injection volume was 150 µl and the concentration was 1 mg/ml.



**Appendix B4.** The HPLC chromatogram of the crude methanol root extract of *D. anomala* from Lesotho. The analytical separation of the extract was done on a C18 reverse phase Phenomenex column at a gradient of 20% - 100% MeCN: H<sub>2</sub>O. The flow rate was 1 ml/min, injection volume was 150 µl and the concentration was 1 mg/ml.

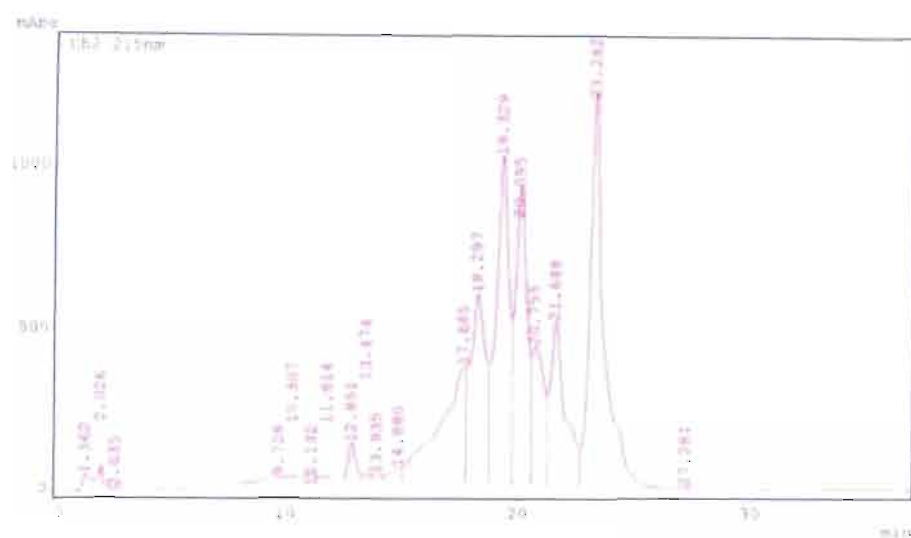


**Appendix B5.** The HPLC chromatogram of the crude petroleum ether root extract of *D. anomala* from Lesotho. The analytical separation of the extract was done on a C18 reverse phase Phenomenex column at a gradient of 20% - 100% MeCN: H<sub>2</sub>O. The flow rate was 1 ml/min, injection volume was 150 µl and the concentration was 1 mg/ml.

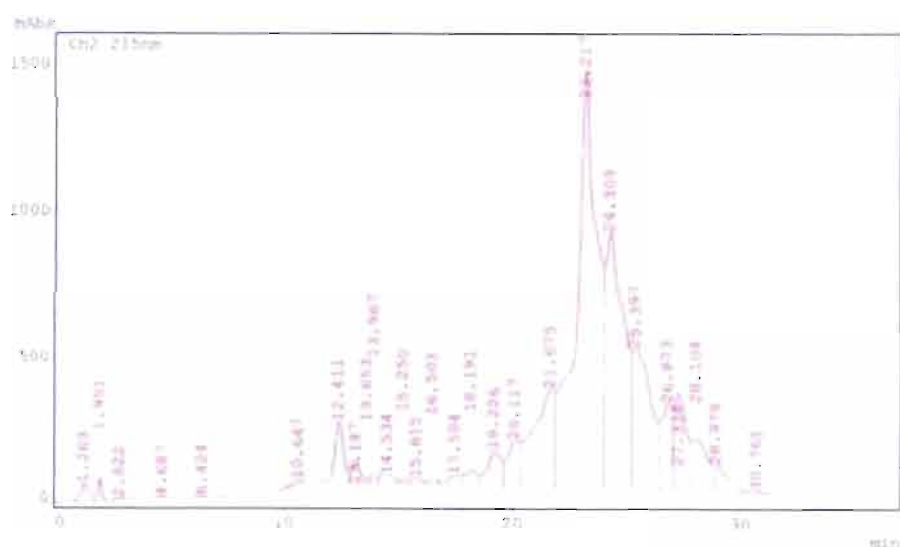
## ***APPENDIX C***



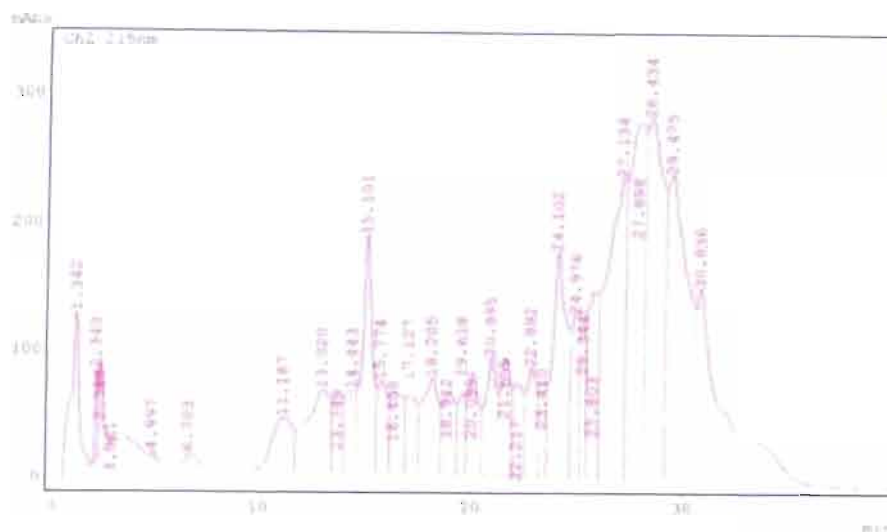
### **THE HPLC CHROMATOGRAMS OF 5% and 1% SPE FRACTIONS**



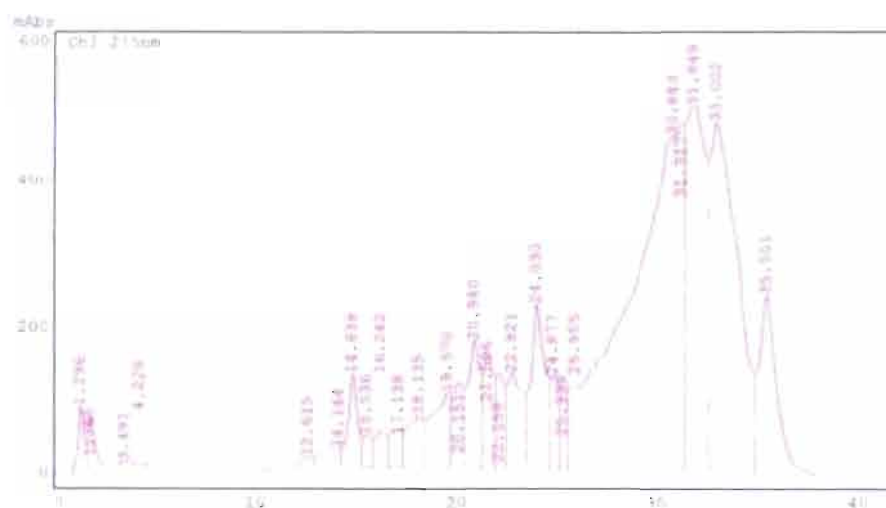
**Appendix C1.** The HPLC chromatogram of fraction 35 from batch I of the cold DCM extract from Lesotho. The analytical separation of the fraction was done on a C18 reverse-phase Phenomenex column (150 x 4.6 mm, 5  $\mu$ m) at a gradient of 30% - 70% MeCN in H<sub>2</sub>O. The flow rate was 1 ml/min, injection volume was 150  $\mu$ l and the concentration was 1 mg/ml.



**Appendix C2.** The HPLC chromatogram of fraction 40 from batch I of the cold DCM extract from Lesotho. The analytical separation of the fraction was done on a C18 reverse-phase Phenomenex column (150 x 4.6 mm, 5  $\mu$ m) at a gradient of 30% - 70% MeCN in H<sub>2</sub>O. The flow rate was 1 ml/min, injection volume was 150  $\mu$ l and the concentration was 1 mg/ml.

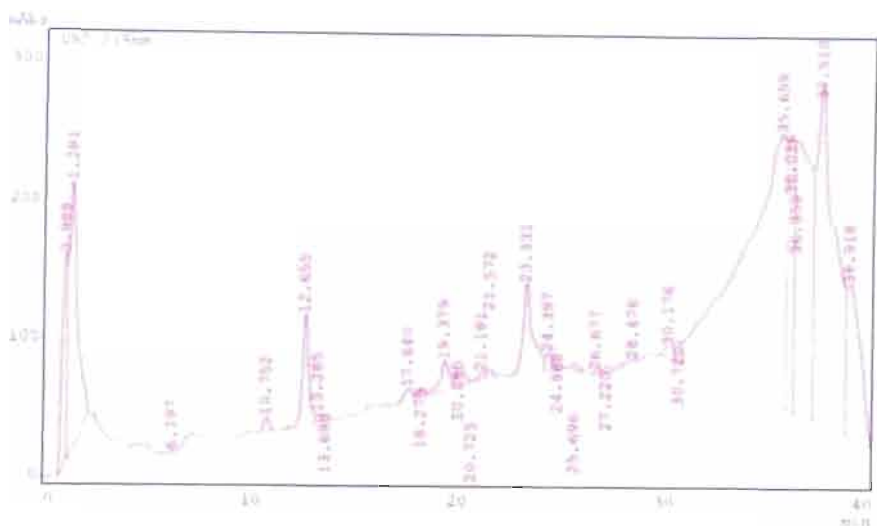


**Appendix C3.** The HPLC chromatogram of fraction 45 from batch I of the cold DCM extract from Lesotho. The analytical separation of the fraction was done on a C18 reverse-phase Phenomenex column (150 x 4.6 mm, 5  $\mu$ m) at a gradient of 30% - 70% MeCN in H<sub>2</sub>O. The flow rate was 1 ml/min, injection volume was 150  $\mu$ l and the concentration was 1 mg/ml.

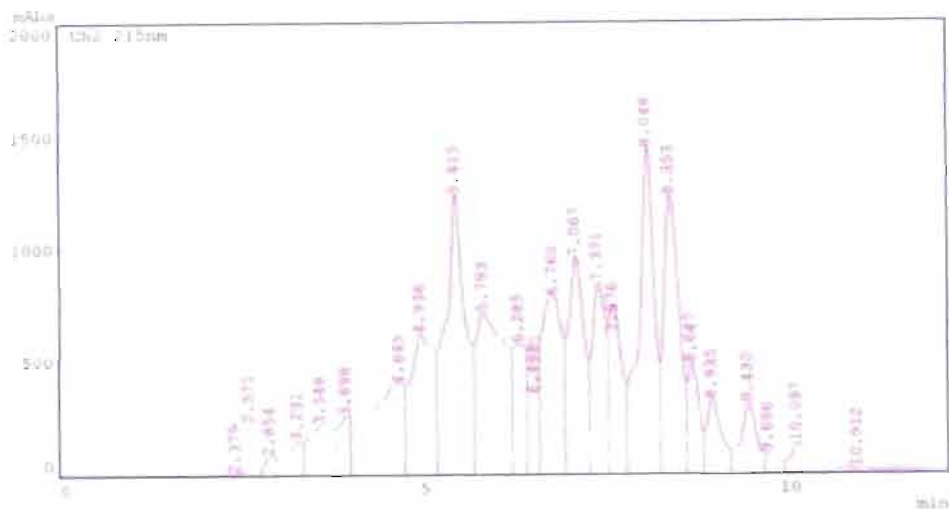


**Appendix C4.** The HPLC chromatogram of fraction 50 from batch I of the cold DCM extract from Lesotho. The analytical separation of the fraction was done on a C18 reverse-phase Phenomenex column (150 x 4.6 mm, 5  $\mu$ m) at a gradient of 30% - 70% MeCN in H<sub>2</sub>O. The flow rate was 1 ml/min, injection volume was 150  $\mu$ l and the concentration was 1 mg/ml.

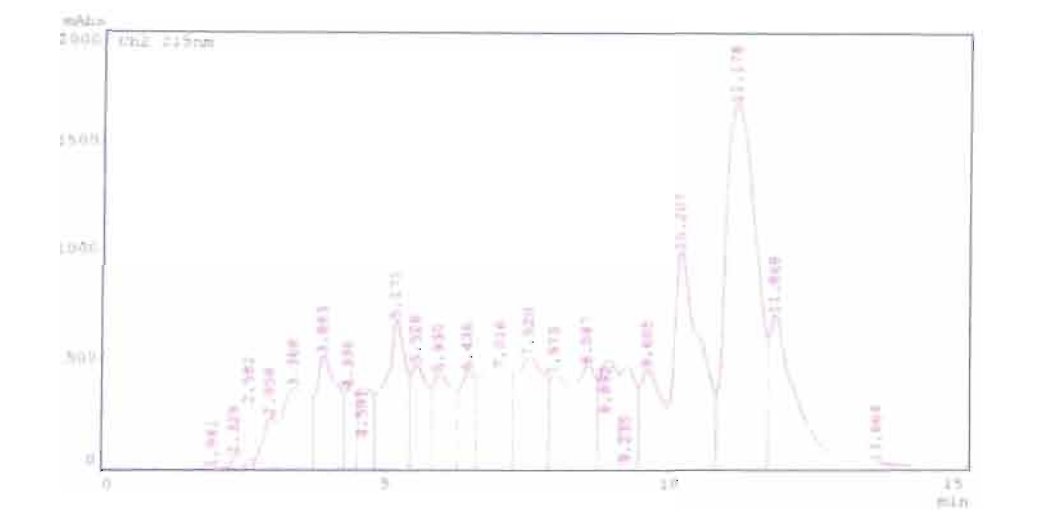




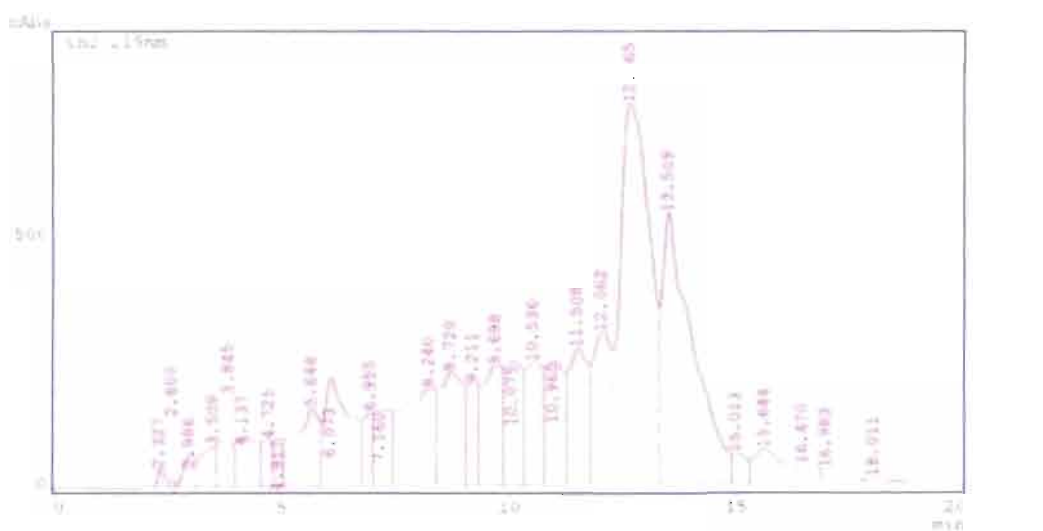
**Appendix C5.** The HPLC chromatogram of fraction 55 from batch I of the cold DCM extract from Lesotho. The analytical separation of the fraction was done on a C18 reverse-phase Phenomenex column (150 x 4.6 mm, 5 µm) at a gradient of 30% - 70% MeCN in H<sub>2</sub>O. The flow rate was 1 ml/min, injection volume was 150 µl and the concentration was 1 mg/ml.



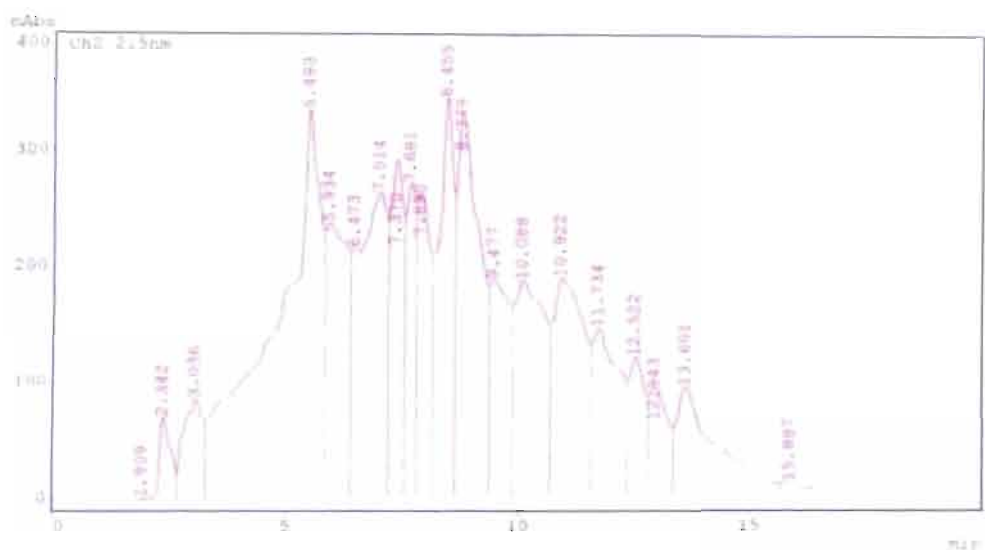
**Appendix C6.** The HPLC chromatogram of fraction 31 from batch I of the cold DCM extract from Lesotho . The analytical separation of the fraction was done on a C18 reverse-phase Haisil column (250 x 4.6 mm, 5 µm) was at a gradient of 50% - 70% MeCN in H<sub>2</sub>O MeCN: H<sub>2</sub>O. The flow rate was 1 ml/min, injection volume was 250 µl and the concentration was 1 mg/ml.



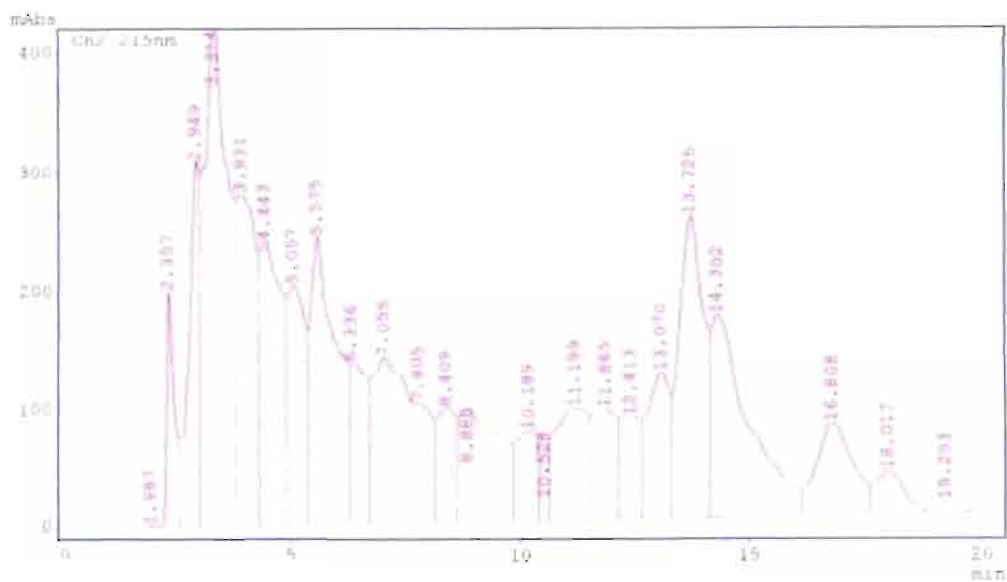
**Appendix C7.** The HPLC chromatogram of fraction 32 from batch I of the cold DCM extract from Lesotho. The analytical separation of the fraction was done on a C18 reverse-phase Haisil column (250 x 4.6 mm, 5 µm) was at a gradient of 50% - 70% MeCN in H<sub>2</sub>O MeCN: H<sub>2</sub>O. The flow rate was 1 ml/min, injection volume was 250 µl and the concentration was 1 mg/ml.



**Appendix C8.** The HPLC chromatogram of fraction 33 from batch I of the cold DCM extract from Lesotho. The analytical separation of the fraction was done on a C18 reverse-phase Haisil column (250 x 4.6 mm, 5 µm) was at a gradient of 50% - 70% MeCN in H<sub>2</sub>O MeCN: H<sub>2</sub>O. The flow rate was 1 ml/min, injection volume was 250 µl and the concentration was 1 mg/ml.



**Appendix C9.** The HPLC chromatogram of fraction 34 from batch I of the cold DCM extract from Lesotho. The analytical separation of the fraction was done on a C18 reverse-phase Haisil column (250 x 4.6 mm, 5 µm) was at a gradient of 50% - 70% MeCN in H<sub>2</sub>O MeCN: H<sub>2</sub>O. The flow rate was 1 ml/min, injection volume was 250 µl and the concentration was 1 mg/ml.

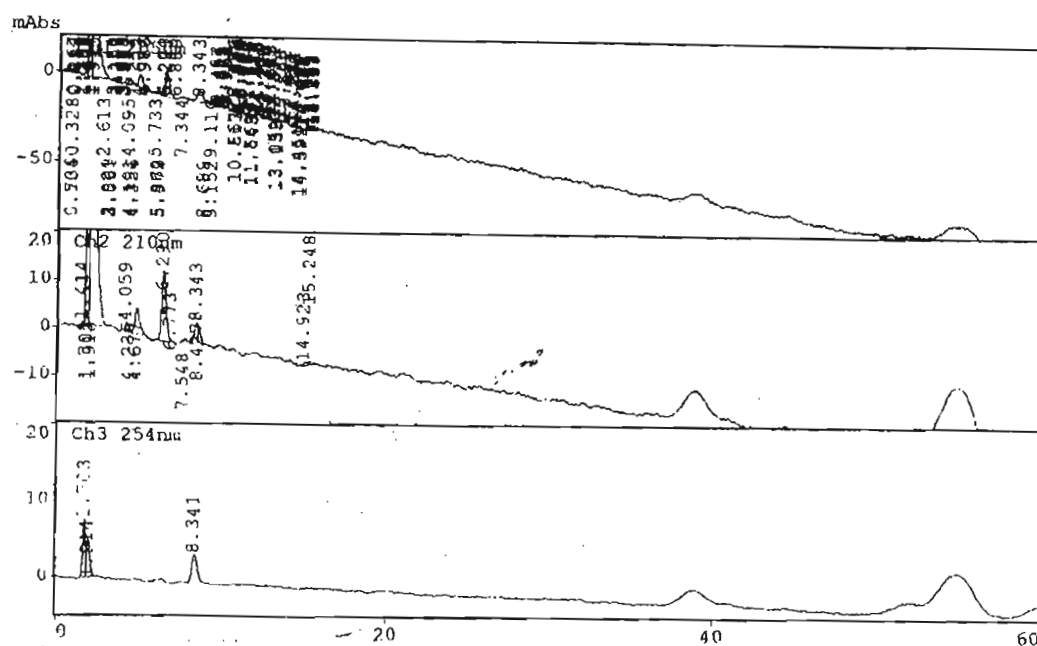


**Appendix C10.** The HPLC chromatogram of fraction 35 from batch I of the cold DCM extract from Lesotho. The analytical separation of the fraction was done on a C18 reverse-phase Haisil column (250 x 4.6 mm, 5 µm) was at a gradient of 50% - 70% MeCN in H<sub>2</sub>O MeCN: H<sub>2</sub>O. The flow rate was 1 ml/min, injection volume was 250 µl and the concentration was 1 mg/ml.

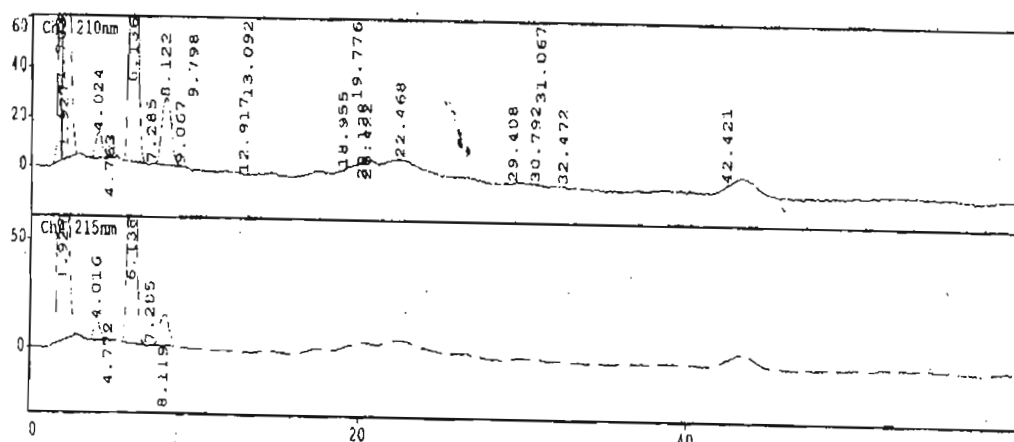
## ***APPENDIX D***



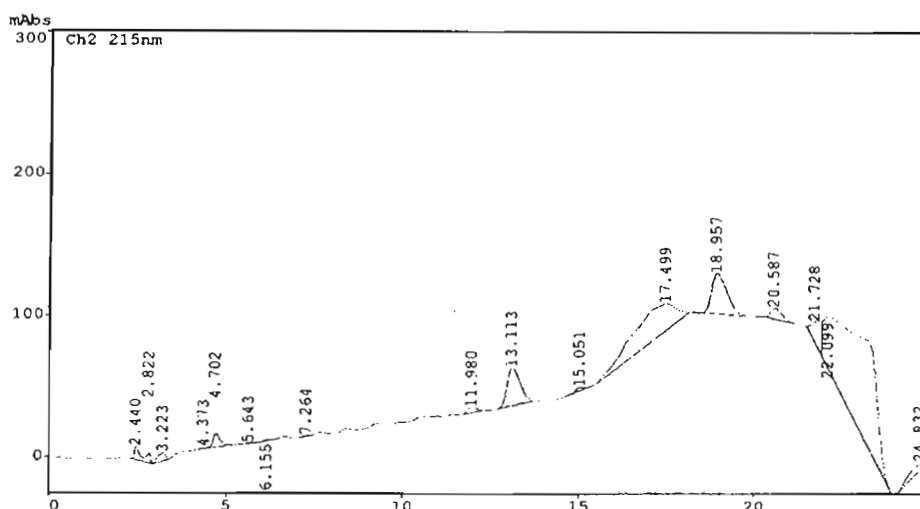
### **THE HPLC CHROMATOGRAMS OF TLC BANDS FROM FRACTION 31**



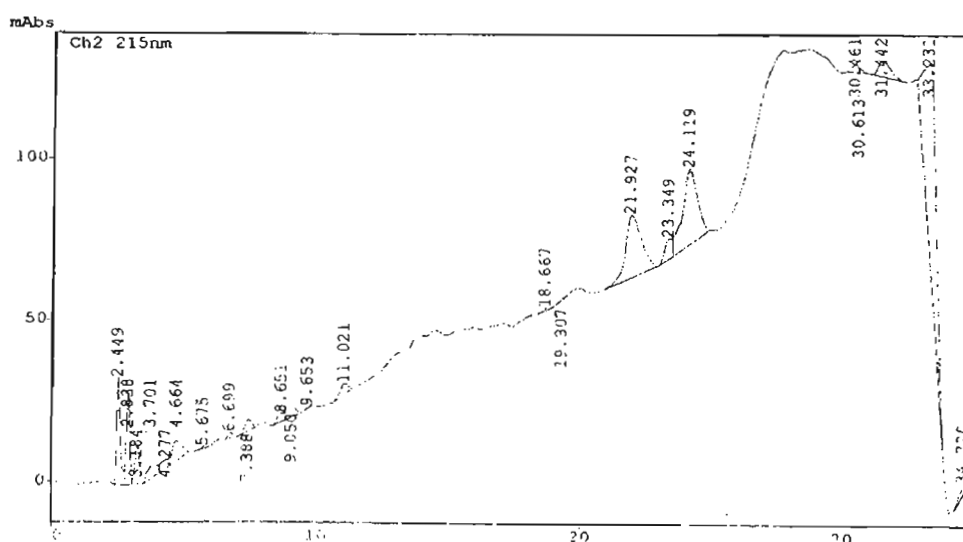
**Appendix D1.** The HPLC chromatogram of a TLC band (band 3, UV = 254 nm) from fraction 31. The fraction was run on a pre-coated aluminium TLC plate (20 x 20 cm silica gel 60F<sub>254</sub> (0.25 mm thickness) and eluted with the benzene: acetic acid (60:40) mobile phase.



**Appendix D2.** The HPLC chromatogram of a TLC band (band 5, UV 254 nm) from fraction 31. The fraction was run on a pre-coated aluminium TLC plate (20 x 20 cm silica gel 60F<sub>254</sub> (0.25 mm thickness) and eluted with the benzene: acetic acid (60:40) mobile phase.



**Appendix D3.** The HPLC chromatogram of a TLC band (band 3, UV = 366 nm) from fraction 31. The fraction was run on a pre-coated aluminium TLC plate (20 x 20 cm silica gel 60F<sub>254</sub> (0.25 mm thickness) and eluted with the benzene: acetic acid (60:40) mobile phase.

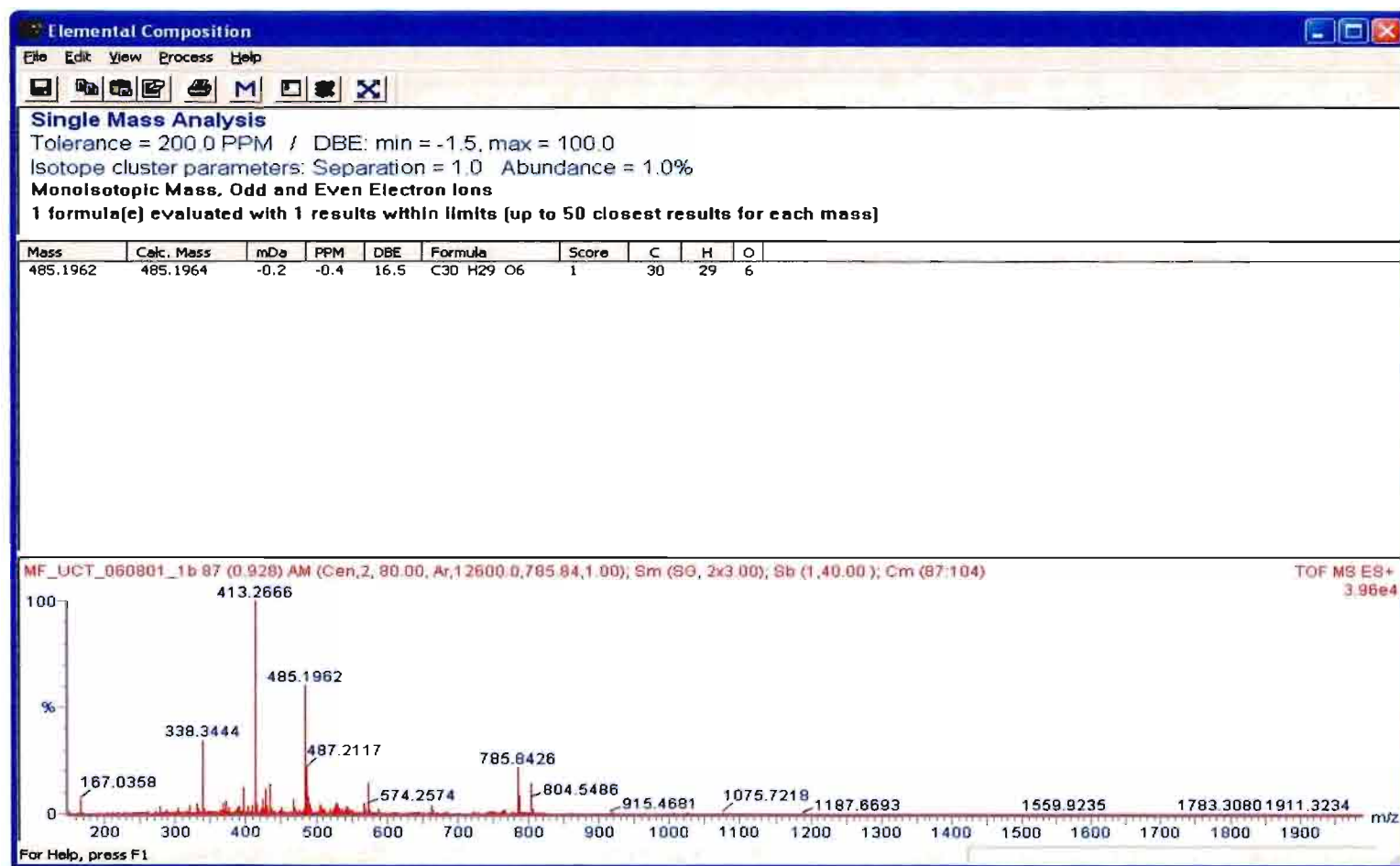


**Appendix D4.** The HPLC chromatogram of a TLC band (band 5, UV = 366 nm) from fraction 31. The fraction was run on a pre-coated aluminium TLC plate (20 x 20 cm silica gel 60F<sub>254</sub> (0.25 mm thickness) and eluted with the benzene: acetic acid (60:40) mobile phase.

## ***APPENDIX E***

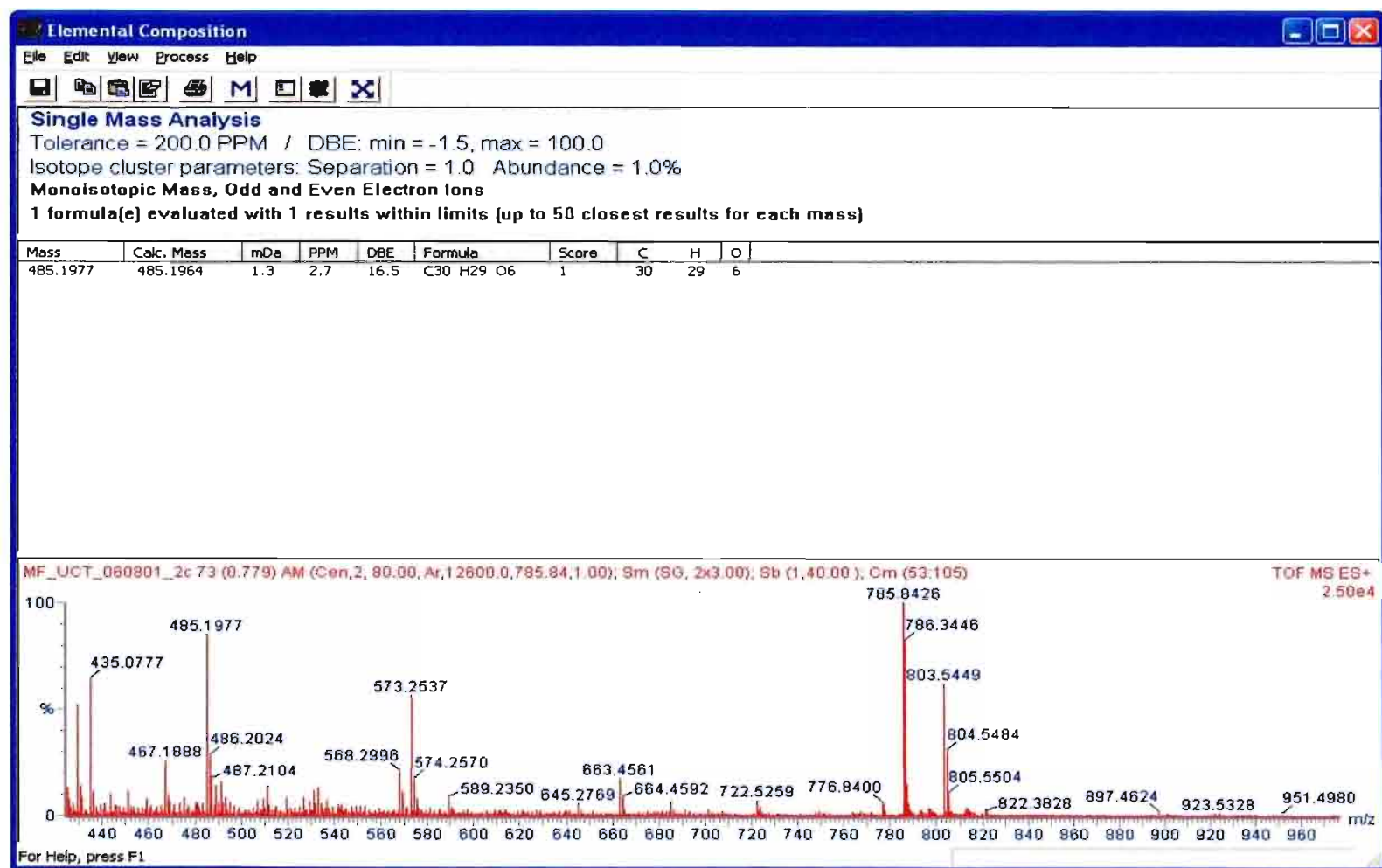
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**HR-MS OF COMPOUNDS MF4 & MF7A and LC-MS OF  
PEAKS PRESENT IN FRACTION 40**

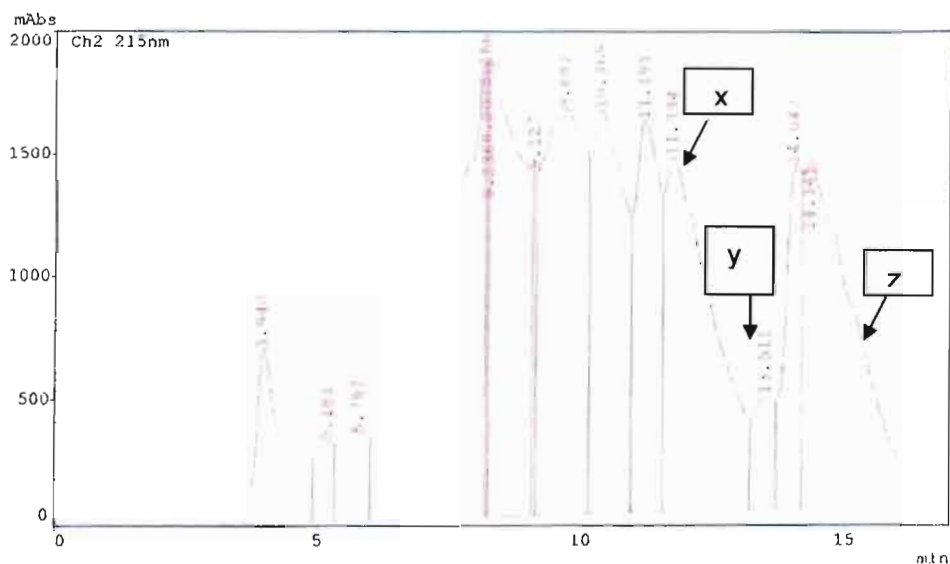


**Appendix E1.** ESI-HRMS showing the experimental and calculated masses and the elemental composition of compound MF4.

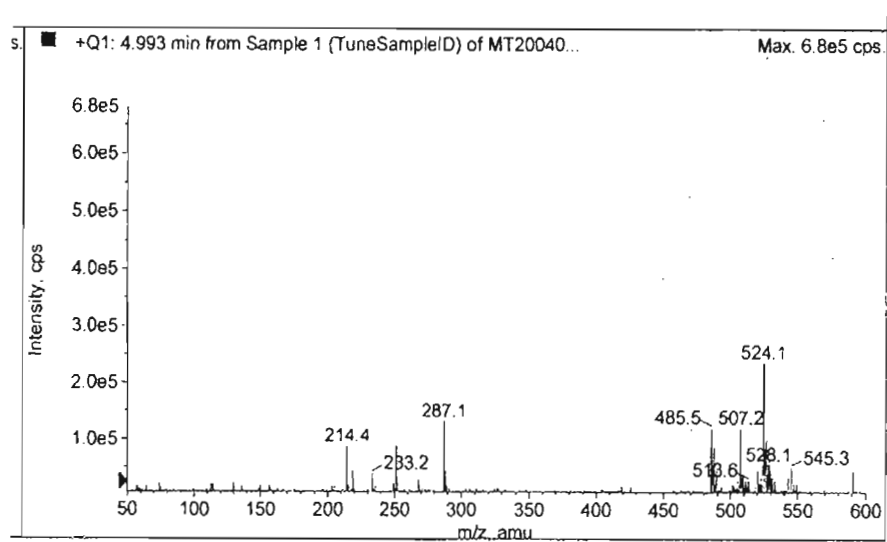




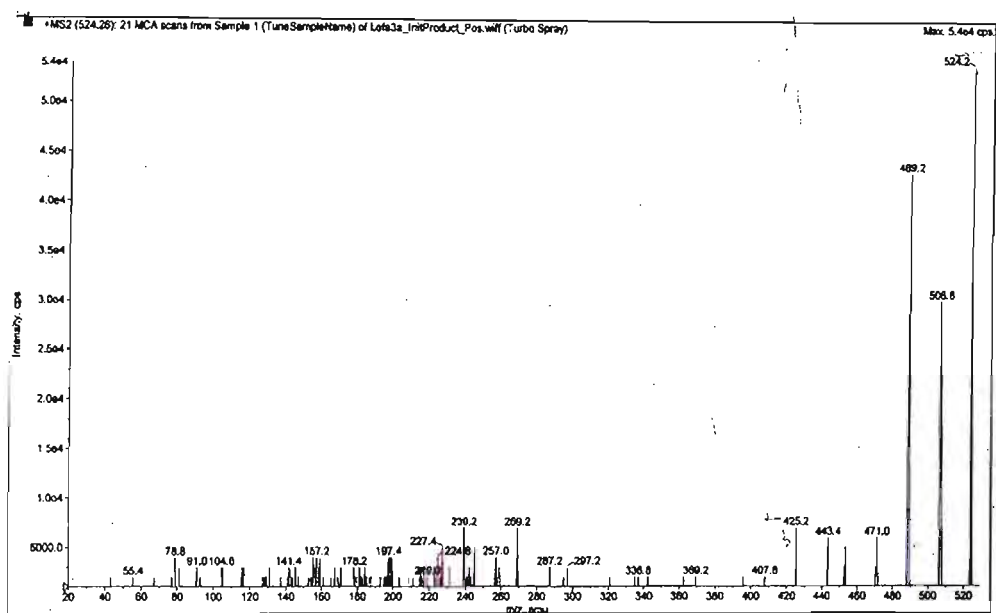
**Appendix E2.** ESI-HRMS showing the experimental and calculated masses and the elemental composition of compound MF7A.



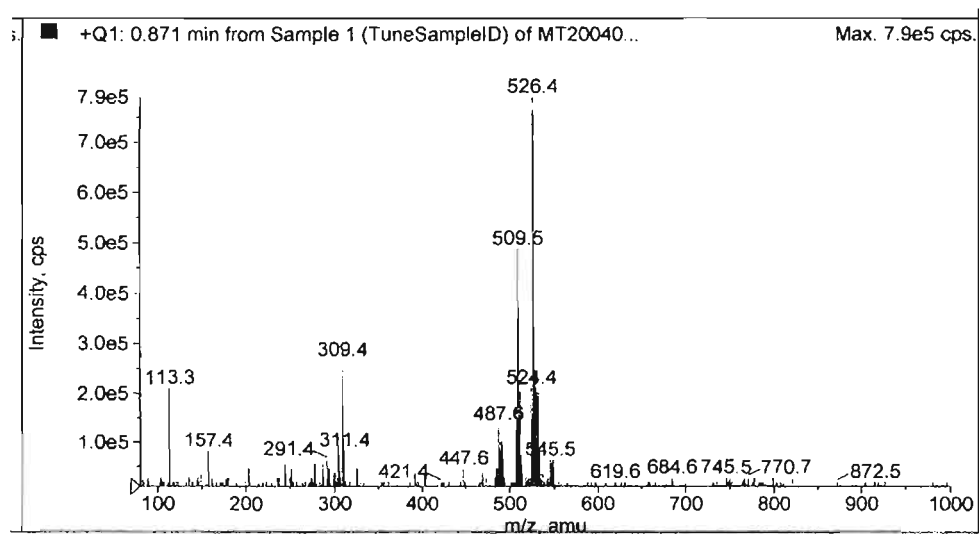
**Appendix E3.** Reverse phase semi-preparative HPLC for fraction 40 showing peaks, MF5 (x), MF6B & MF6E (y) and MF8 (z). The fraction was ran at an isocratic elution of 60% MeCN in H<sub>2</sub>O on a Haisil column (250 x 10 mm, 5  $\mu$ m), the concentration was 4.5 mg/ml, flow rate was 2.0 ml/min and the injection volume was 150  $\mu$ l.



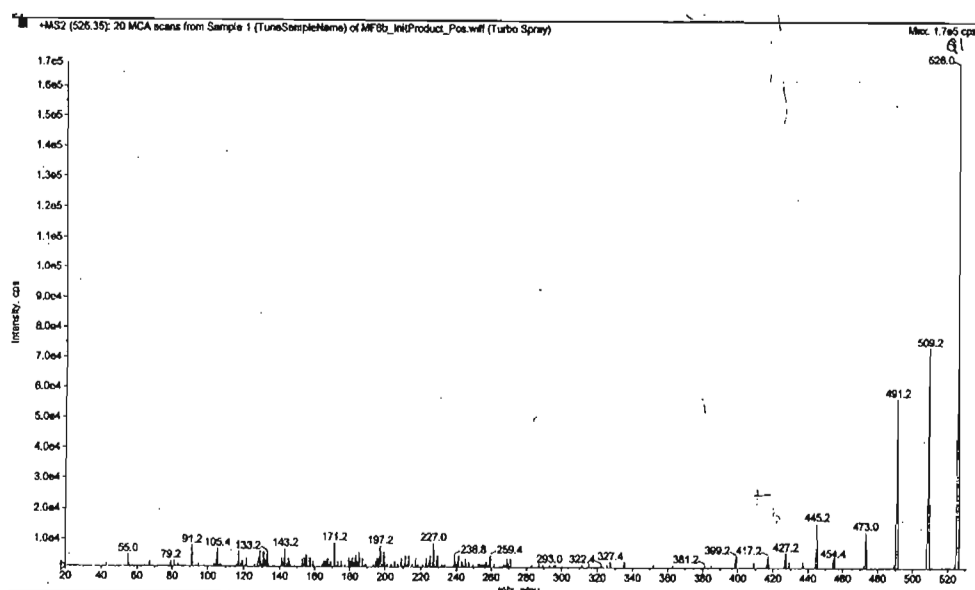
**Appendix E4a.** LC/MS of peak MF5 (m/z 524).



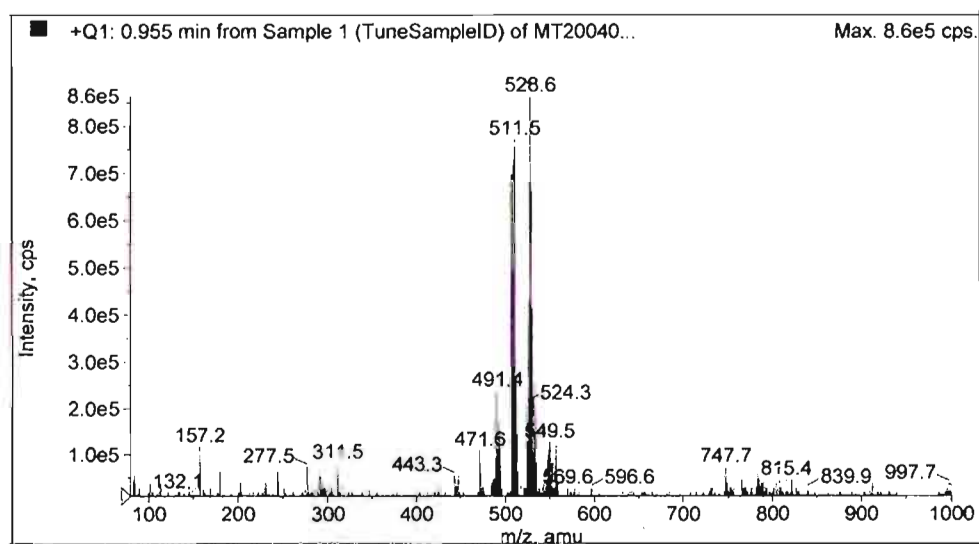
#### Appendix E4b. APCI of peak MF5.



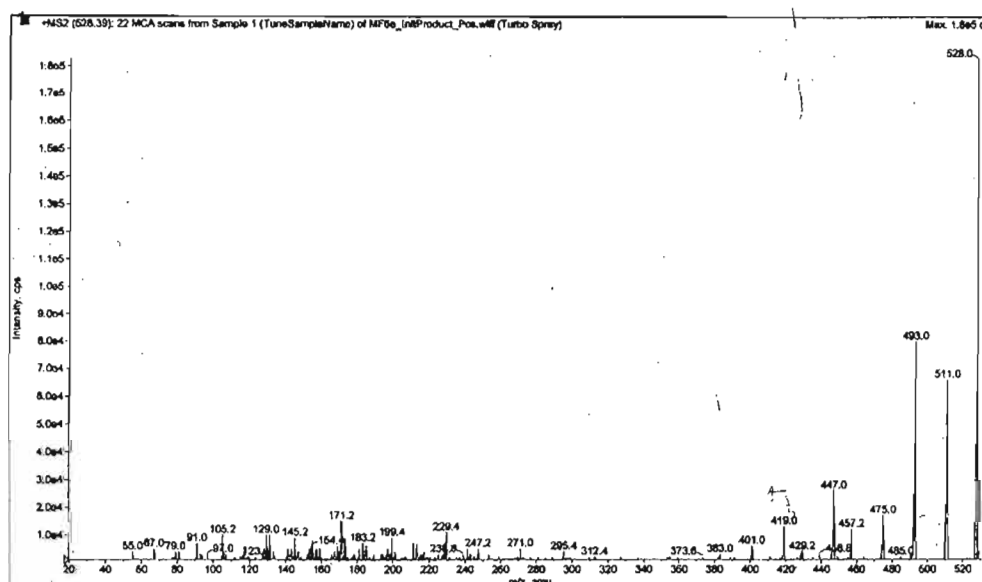
#### Appendix E5a. LC/MS of peak MF6B (m/z 526).



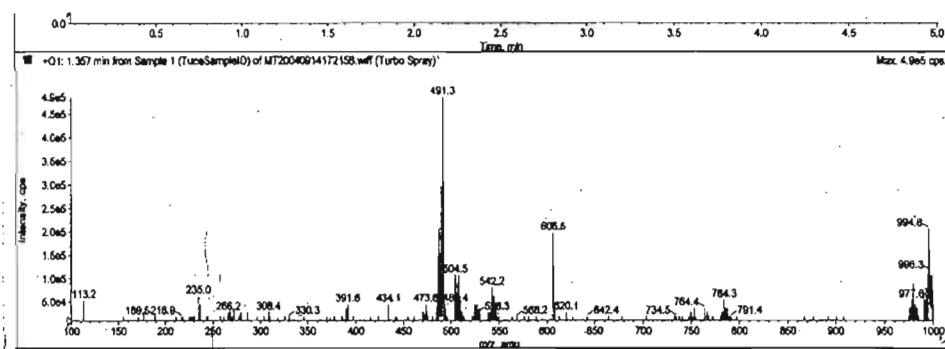
# Appendix E5b. APCI of peak MF6B.



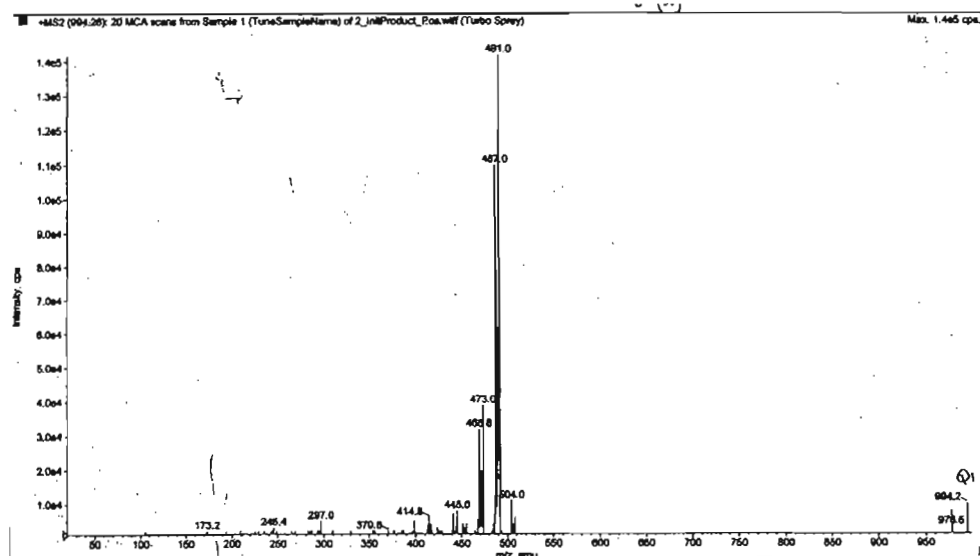
# Appendix E6a. LC/MS of peak MF6E (m/z 528).



Appendix E6b. APCI of peak MF6E.



Appendix E7a. LC/MS of peak MF8 (m/z 491).

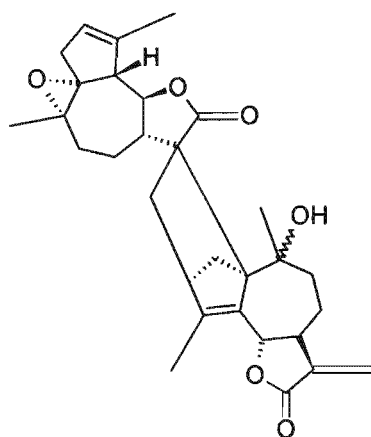


Appendix E7b. APCI of peak MF8.

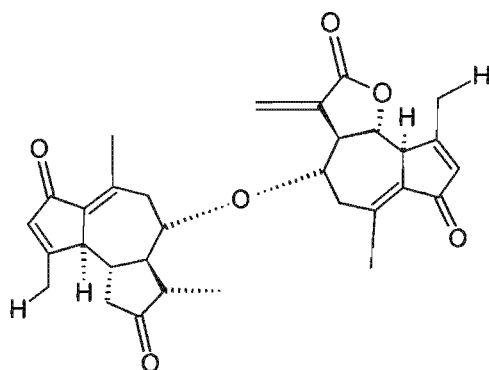
## ***APPENDIX F***

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**CHEMICAL STRUCTURES OF DIMERIC  
GUAIANOLIDES SHOWING THE DIFFERENT LINKS  
BETWEEN THE TWO MONOMERS**

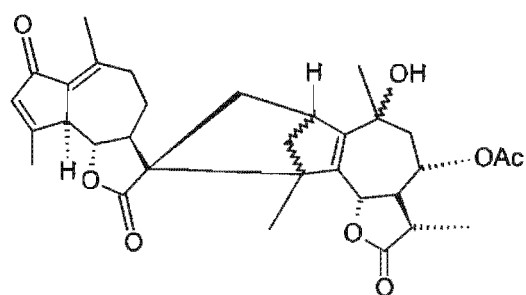


**Appendix F1.** Dimeric guaianolide isolated from the aerial parts of *Artemisia myriantha*. The molecular weight of the compound could not be directly determined by mass spectrometry [Wong & Brown, 2002].



**Appendix F2.** Dimeric guaianolide,  $m/z$  503  $[M-H]^-$  isolated from *Lactuca indica* [Hou et al., 2003].





**Appendix F3.** Dimeric guaianolide,  $m/z$  550  $[M]^+$  isolated from the flower heads of *Achillea collina* [Trendafilova et al., 2006].